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Woodland Strawberry (*Fragaria vesca)* Summer and Winter Leaf Development, Stolon Production and Leaf Pigments in Twelve European Genotypes under Different Temperature Treatments

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Master's Thesis
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Tiivistelmä – Referat – Abstract

Ecophysiology and ecology in plants are strongly affected by the conditions surrounding them. Adaptation aids plants to survive and to succeed in the prevailing conditions. Winter is a challenge to plants, particularly in northern latitudes and higher altitudes, because it exposes plants to cold and drought, for example. Plants survive from winter on species level with the help of genetic adaptations and as individuals also with the help of acclimation. Woodland strawberry (*Fragaria vesca)* has been observed to grow separate winter leaves. This allows it to continue photosynthesis in mild conditions during winter, thus improving its energy balance, and to start growing earlier than other species in the spring, which is beneficial in interspecific competition. *Fragaria vesca* is a species that has wide distribution in the northern hemisphere, and its genotypes are found from very different locations and conditions. However, adaptive traits such as producing a new set of leaves for winter can turn out to be a disadvantage if environmental conditions change rapidly. Climate change brings about changes that are difficult to predict, and these changes are advancing at a fast pace when compared to the developmental history of plants.

The aim of this thesis was to study the effect of temperature on summer and winter leaf development, stolon formation and summer and winter leaf chlorophyll, flavonol and anthocyanin content in different *Fragaria vesca* genotypes. Leaf chlorophyll and secondary compound content give information about leaf development and stress reactions in plants. Plants are known to produce anthocyanins in order to protect the photosynthetic apparatus during chlorophyll recovery in leaf senescence. Anthocyanins are also produced as a response to low temperatures. Research increases knowledge of the ecophysiological and winter ecology-related processes in *Fragaria vesca* and in the commercially valuable Rosacea-family as well as provides information about the possible responses of these organisms to climate change.

Material for the study consisted of twelve European *Fragaria vesca* genotypes, which had originally been collected from five countries: Norway, Finland, Germany, Italy and Spain. The genotypes had been collected from different latitudes, and they also expressed altitudinal differences. In this study, these genotypes were kept in two temperature treatments, warm (+16°C) and cold (+11°C/six weeks, after which +6°C/four weeks) at a greenhouse. Leaf development was studied by measuring summer and winter leaf middle leaflet width and length, and petiole length. Stolons from each plant individual were counted on a weekly basis and observations about stolon production in relation to the timing of summer leaf senescence and winter leaf development were made at the same time. Leaf chlorophyll and secondary compound content was measured with a Dualex-meter, which provided values for chlorophyll, flavonol and anthocyanin content. The underlying assumption was that cold temperature would induce winter leaf development and summer leaf senescence.

The results show that there were differences in summer leaf size between genotypes. Winter leaves had differences between genotypes, but also within genotypes at different temperature treatments. Stolon count was lower and stolon production ceased slightly earlier in the cold treatment. Moreover, summer leaf chlorophyll content decreased in both treatments, but the summer leaves senesced earlier in the warm room. Summer leaf flavonol and anthocyanin values were generally higher in the cooler temperature treatment. Anthocyanins were also produced by winter leaves in the cooler temperature treatment.

Based on the results, *Fragaria vesca* genotypes had differences related to their origin, but temperature also had an effect on winter leaf development, stolon production and the production of secondary compounds. The effect of cold temperature on the size of developing winter leaves was clear. In the cooler temperature treatment, the winter leaves were smaller than in the warmer treatment. The anthocyanin content of summer leaves was higher than in the winter leaves, and the summer leaf anthocyanin content was higher in the colder temperature treatment, where the stress related to the photosynthetic apparatus and low temperatures was combined. Nevertheless, lower temperature did not explain all the responses observed in the genotypes of the study, and thus it is likely that acclimation and winter leaf development in *Fragaria vesca* are affected by some other factor in addition to temperature, e.g. light regime. A possible continuation for this work would be to study the effect of light conditions or milder winters on winter leaf development in *Fragaria vesca* genotypes and on the physiology of the species.

Avainsanat – Nyckelord – Keywords
***Fragaria vesca*, winter ecology, adaptation, acclimation, chlorophyll, anthocyanin, flavonol, ecophysiology**

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This thesis is part of a larger research collaboration between the Plant Ecophysiology and Climate Change Group, Faculty of Biological and Environmental Sciences and the Strawberry Research Group, Faculty of Forestry and Agriculture, University of Helsinki, Finland. As part of this collaboration, the aim is to publish the results of this study in a scientific journal.

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Kahdentoista eurooppalaista alkuperää olevan ahomansikkagenotyypin *(Fragaria vesca)* kesä- ja talvilehtien kehitys, rönsyjen tuotanto ja lehtien pigmentit eri lämpötilakäsittelyissä

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Tiivistelmä – Referat – Abstract

Kasvien ekofysiologiaan ja ekologiaan vaikuttavat vahvasti niitä ympäröivät olosuhteet. Sopeutuminen vallitseviin olosuhteisiin auttaa kasveja selviytymään ja menestymään. Talvi on kasveille haaste, erityisesti pohjoisilla leveysasteilla ja vuoristoisilla alueilla, sillä se altistaa kasvit mm. kylmyydelle ja kuivuudelle. Lajitasolla kasvit selviytyvät talvesta geneettisesti erilaisten sopeutumien avulla sekä yksilötasolla vuosikierrossaan myös akklimaation avulla. Ahomansikan (*Fragaria vesca)* on havaittu kasvattavan talven ajaksi erilliset talvilehdet, joiden avulla kasvi pystyy jatkamaan yhteyttämistä leudoissa olosuhteissa talven aikana. Tämä parantaa sen energiatasapainoa sekä sallii sen aloittaa kasvun muita lajeja aiemmin keväällä, mikä on eduksi lajien välisessä kilpailussa. Laji on myös levinnyt laajalle alueelle pohjoisella pallonpuoliskolla, jossa sen eri genotyyppejä tavataan hyvin erilaisista olosuhteista. Sopeutumisen mukanaan tuomat erityispiirteet kuten erillisten talvilehtien tuottaminen voivat kuitenkin muuttua haitalliseksi, mikäli olosuhteet muuttuvat nopeasti. Ilmastonmuutos tuo mukanaan vaikeasti ennustettavia muutoksia, jotka etenevät kasvien kehityshistorian näkökulmasta nopeassa tahdissa.

Työn tavoitteena oli tutkia lämpötilan vaikutusta eri ahomansikkagenotyyppien kesä- ja talvilehtien kehitykseen, rönsyjen muodostukseen sekä kesä- ja talvilehtien klorofylli-, flavonoli- ja antosyaanipitoisuuteen. Lehtien klorofylli- ja sekundääriyhdisteiden pitoisuudet antavat tietoa lehtien kehityksestä ja stressireaktioista. Antosyaaneja tuotetaan suojelemaan fotosynteesikoneistoa klorofyllin talteenoton ja lehtien kuoleman yhteydessä, ja niitä kehittyy myös vasteena aleneviin lämpötiloihin. Tutkimuksen avulla saadaan tietoa ahomansikan ja taloudellisesti arvokkaiden Rosaceae-suvun lajien ekofysiologisista prosesseista, erityisesti talviekologian kannalta, sekä näiden kasvien mahdollisista vasteista ilmastonmuutokseen.

Tutkielman aineisto koostui kahdestatoista eurooppalaisesta ahomansikkagenotyypistä, jotka oli alun perin kerätty viidestä maasta: Norja, Suomi, Saksa, Italia ja Espanja. Alkuperät oli kerätty eri leveysasteilta, ja myös niiden kasvupaikat sijaitsivat eri korkeuksilla. Tutkimuksessa koekasveja pidettiin kasvihuoneessa kahdessa eri lämpötilakäsittelyssä, lämpimässä (+16°C) ja viileässä (+11°C/kuusi viikkoa, jonka jälkeen +6°C/neljä viikkoa). Kokeen aikana lehtien kehitystä tutkittiin mittaamalla kesä- ja talvilehdistä keskilehdykän leveys ja pituus sekä lehtiruodin pituus. Koekasvien rönsyt laskettiin viikoittain ja samalla seurattiin rönsyjen tuotantoa suhteessa kesälehtien lakastumiseen ja talvilehtien kehitykseen. Lehtien klorofylliä ja sekundääriyhdisteitä mitattiin Dualex-mittarilla, jolla saatiin arvot klorofylli-, flavonoli- ja antosyaanipitoisuudesta. Taustaoletuksena oli, että kylmä lämpötila olisi talvilehtien kehitykseen ja kesälehtien lakastumiseen vaikuttava tekijä.

Tulokset osoittivat, että kesälehtien koossa oli eroja eri genotyyppien välillä. Talvilehdillä eroja oli tämän lisäksi myös saman genotyypin sisällä eri lämpötilakäsittelyissä. Rönsyjä kehittyi viileässä lämpötilassa vähemmän ja niiden tuotanto lakkasi hieman aiemmin kuin lämpimässä. Kesälehtien klorofyllipitoisuus laski molemmissa lämpötilakäsittelyissä, ja kesälehdet lakastuivat aiemmin lämpimässä huoneessa. Kesälehtien flavonoli- ja antosyaanipitoisuudet olivat keskimäärin korkeampia viileämmässä lämpötilakäsittelyssä. Antosyaaneja kehittyi myös talvilehdillä viileässä lämpötilassa.

Johtopäätöksenä voitiin todeta, että eri genotyyppien välillä on niiden alkuperästä johtuvia eroja, mutta lisäksi lämpötila vaikuttaa talvilehtien kehitykseen, rönsyjen muodostumiseen ja sekundääriyhdisteiden tuotantoon. Kehittyvien talvilehtien kokoon lämpötilalla oli selkeä vaikutus siten, että alhaisemmassa lämpötilassa lehdistä kehittyi pienempiä genotyypin alkuperästä riippumatta. Antosyaaneja kehittyi kesälehdissä enemmän kuin talvilehdissä, ja kesälehdillä niiden pitoisuus oli korkeampi viileämmässä lämpötilakäsittelyssä, jossa yhdistyivät fotosynteesikoneistoon liittyvä stressi ja kylmyys. Alhaisempi lämpötila ei kuitenkaan selittänyt kaikkia tutkimuksessa tarkasteltuja kasvien kehitykseen liittyviä eroja. Niinpä on todennäköistä, että ahomansikoiden talveentumiseen ja talvilehtien kehittymiseen vaikuttaa lämpötilan ohella jokin muu tekijä, esim. valo-olosuhteet. Jatkona työlle olisi mahdollista tutkia valo-olosuhteiden tai lämpenevien talviolosuhteiden vaikutusta *Fragaria vesca* -genotyyppien talvilehtien kehitykseen ja lajin fysiologiaan.

Avainsanat – Nyckelord – Keywords
***Fragaria vesca*, talviekologia**, **sopeutuminen, akklimaatio, klorofylli, antosyaani, flavonoli, ekofysiologia**

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Muita tietoja – Övriga uppgifter – Additional information
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All images presented in this thesis have been taken by the author.

1. Introduction

Woodland strawberry, *Fragaria vesca* L., is a perennial herb of the Rosaceae family, native to Europe and Asia with current natural distribution extending around the northern hemisphere (Kew Species Profiles 2020). This rosette-forming species can be found growing in a variety of habitats, and it reproduces both sexually with seeds and vegetatively with clones. It is also a plant that develops a new set of leaves for winter in the fall while the summer leaves wither and die. Winter leaves allow *Fragaria vesca* to remain photosynthetically active late in the fall, early in the spring and during warm periods in winter (Åström et al. 2015). Thus, the plant can control its energy balance during winter and start growing earlier in spring than other species without winter leaves. In interspecific competition this is, of course, a great benefit.

Since *Fragaria vesca* is such a widely spread species, there is great diversity within it. This diversity is both genetic and phenotypic. As with plants in general, the phenotype of *Fragaria vesca* is linked to local conditions. For example, research has shown that as a result of morphological plasticity, plants in marginal conditions such as cold habitats, where growing season is short, tend to be smaller in size than individuals of the same species located in more favorable conditions (Crawford 2014). Inspecting the natural diversity within the species allows us to better understand which traits can be beneficial in certain environments and act as adaptations to specific conditions (Hilmarsson et al. 2017).

Based on previous research concerning this species, it is known that the summer and winter leaves of *Fragaria vesca* are produced at different times during the year, a phenomenon referred to as seasonal leaf dimorphism (Åström et al. 2015). However, the temperature-relatedness of winter leaf development as well as the effect of temperature on winter leaf development in different genotypes of *Fragaria vesca* has remained unclear. Related to this is also how geographically different genotypes of *Fragaria vesca* respond to various temperature conditions at a general level.

The species in question, *Fragaria vesca*, is an important object for research because its genome is known and because its relative, the cultivated strawberry, is commercially highly valuable. Studying *Fragaria vesca* helps in understanding the mechanisms of strawberry plants in general and enables the development of better cultivation. Furthermore, the results can also give cues about how similar processes might take place in other plant species that are related to *Fragaria vesca*, especially in the Rosaceae family.

In addition to cultivation-related aspects, studying *Fragaria vesca* is also justifiable because it is a plant with a wide distribution across several latitudes in the northern hemisphere (Kew Species Profiles 2020), which will be considerably affected by climate change. Proposed climatic scenarios differ, but generally it seems clear that climate change will alter the environment for all living organisms as well as the framework for plant cultivation. The winter leaf development of *Fragaria vesca* is apparently an adaptation to winter conditions, but the question is what will happen if or when these conditions change? Moreover, since there are so many

different genotypes of *Fragaria vesca*, do they all respond in the same way? The topic is important because it offers information on how climate change will affect different *Fragaria vesca* genotypes and perhaps also the plant communities in which they occur in the wild.

This study focuses on a single species, *Fragaria vesca*, but at the same time also covers and combines several aspects of plant biology. The measurements conducted on the *Fragaria vesca* are connected to plant morphology as well as plant primary and secondary compounds. By focusing on several genotypes of the same species from different areas, plant diversity and distribution are also considered. The focus on winter leaves reveals *Fragaria vesca* interactions with their physical environment, which links the topic into plant ecology and, in the case of this study, more specifically into winter ecology. On a larger scale, the ties to ecology and studying adaptations to environmental conditions connect the entire study to the discipline of ecophysiology.

1.1 Plant Winter Ecology

In general, winter is a challenge that plants living in high latitudes and other cold areas of the world must face and overcome. The variety of strategies, responses and their combinations is comparable to the tremendous variety of different plant species. Some species, the annuals, overwinter only as seeds, but many others, the perennials, must survive in very different conditions compared to milder seasons. Moreover, in some areas the ranges of temperature that the plants experience on an annual basis is very wide, from tens of degrees below freezing to tens of degrees above freezing. Naturally, this requires adjustments.

When discussing plant ecology at large and winter ecology in particular, two important concepts to note are adaptation and acclimation. Acclimation is a mechanism that allows individual plants in cold climates to resist the harmful effects of freezing. This is accomplished by gradual structural and metabolic changes, for example in cell membrane structures and photosynthesis levels, that are usually induced by cooling temperatures and changes in light availability. This process is sometimes also called ‘hardening’. The changes are reversible, which allows the plants not only to survive from winter, but to resume growth and make the most of more favorable conditions once spring arrives. (Crawford 2014, Marchand 2014) The acclimation process is usually accompanied by the emergence of specific antifreeze proteins (AFPs) that act in preventing potential damages caused by ice crystals (Crawford 2014).

Adaptation, in turn, is a phenomenon that occurs on a genetic level and is a response to changes in the environment that persist for longer periods of time than mere seasonal fluctuations (Norman et al. 1998). Adaptation in plants requires several generations and comes in two subtypes, which are capacity adaptation and functional adaptation. Capacity adaptation allows plants to complete their metabolic processes even under harsh conditions by increasing enzyme concentrations, for example. Functional adaptation or

adjustment refers to phenotypic plasticity as a response to stress. A third term, acclimatization, refers to processes that also take place on a genetic level, over several generations. Thus, acclimatization is a type of adaptation to cold. (Crawford 2014) However, it should be noted that the definition of acclimatization by Crawford (2014) is not followed by all authors and publications. Adaptation is beneficial to plants, but it can have its negative effects too. High level of adaptation to specific conditions can increase inability to cope with rapid environmental changes (Crawford 2008). Such rapid changes might occur in the future as a consequence of climate change.

Winter poses many threats to plants, and their specificity affects the overwintering strategy of an individual species. For perennial plants, the main threats are low temperatures and drought, which both cause stress and are linked to each other (Marchand 2014). Desiccation is a likely threat in winter, particularly if there is no protective layer of snow covering the plant. Dehydration can be caused by many different factors separately and jointly. For example, wind and solar radiation may increase transpiration rates while the plants cannot recover lost water from frozen ground (Salonen 2006).

Low temperatures are normally a threat to plants because their cells contain large amounts of water. If water freezes, it forms ice crystals that cause mechanical injury to cell structures (Chalker-Scott 1999). Ice crystals can form either inside the cells, which is usually fatal (Marchand 2014) or in extracellular spaces (Chalker-Scott 1999), which is when they can target the plasma membrane (Yamazaki et al. 2009). Extracellular ice formation usually takes place first and acts as a barrier to intracellular freezing by drawing water out of the cell, enlarging the extracellular ice crystal and at the same time increasing the concentration of intracellular solvents, which in turn decreases the cell's freezing point (Marchand 2014). However, when extracellular water turns to ice, it prevents both access to water and water movement within the plant. Moreover, when cells lose water, they become dehydrated, and thus freezing is connected to desiccation.

In addition to threatening plants with desiccation, winter can also affect plants by preventing them from acquiring nutrients, oxygen and sometimes even light. Access to nutrients can be prevented by frozen soil, ice-encasement can affect oxygen availability, and possible snow cover can block light supply. In fact, two particularly difficult threats for plants in cold climates are freeze-thaw cycles and ice-encasement, where the entire plant or significant parts of it become covered by a layer of ice. This happens, for example, during mild winters when rain falls and temperatures fluctuate around freezing point. Ice-encasement causes anoxia and if the plants are able to survive from it, the next threat is post-anoxic injury, which often happens when temperatures increase rapidly, and plants are suddenly reconnected with oxygen supply. (Crawford 2014) Freeze-thaw cycles in winter can also be a great threat to plants acclimated to cold winter conditions. Adjustments to sudden warm temperatures may be delayed, and once they have been completed, the following danger may be injuries caused by sudden refreezing (Marchand 2014).

An important environmental factor in winter ecology is also snow, or these days often the lack of it. Snow has a dual effect on plants in the winter. On one hand, snow cover creates a warmer microclimate for plants (Marchand 2014). Moreover, under snow the fluctuations in environmental conditions, particularly in light intensity and temperature, are less extreme (Solanki et al. 2019). On the other hand, this warmer environment combined with moisture can also increase the likelihood of fungal disease infections, and late snowmelt in areas where the growth season is short to begin with can reduce the time that plants have to complete their reproductive cycles (Salonen 2006). The winter leaves of *Fragaria vesca* guarantee an early start in spring development compared to other plant species that must first grow new leaves in order to start photosynthesizing.

For plants with a distribution across several latitudes, which is the case of *Fragaria vesca*, the nature of winters varies greatly within the species too. Thus, in addition to differences between species, there are also differences within species in levels of adaptation to different stresses, since maintaining the ability for all kinds of responses would be energetically expensive. Being able to adapt sufficiently, but not excessively has a direct effect on whether the plant survives or fails to do so.

1.2 *Fragaria vesca* Characteristics and Leaf Life Span in Plants

In terms of access to light and leaf function, *Fragaria vesca* is a small understory species, a characteristic that is reflected in its growth form (Jurik & Chabot 1986). Moreover, it is a species that expresses seasonal leaf dimorphism, a trait that is considered to be an adaptation to winter. The two sets of leaves produced by *Fragaria vesca* have distinctive qualities. Generally, the winter leaves are smaller, have more hairs, more stomata and denser mesophyll (Åström et al. 2015). *Fragaria vesca* also has more than one strategy for reproduction: the formation of seeds, and vegetative reproduction by forming side branches (axillary shoots) beneath the apex of the rosette and stolons with ramets. However, having multiple strategies for reproduction is both a benefit and a tradeoff for resources (Van Drunen & Dorken 2012).

When it comes to winter and perennial plant leaves, there are surprisingly many strategies. A simplified view would be to think that perennials, both trees and smaller plants, either shed their leaves in the fall or stay evergreen and renew their leaves only as they get older. This is not the entire truth, however. In the northern hemisphere species such as *Oxalis acetosella* can be classified as winter-green because it keeps its leaves throughout the winter and produces a new set in the following spring or early summer (Tessier 2004). This same strategy is followed by *Hepatica nobilis*. *Fragaria vesca* in turn produces a new set of leaves specifically for winter. Leaf exchange strategy in higher plants has developed to what is most advantageous for each species' carbon balance in the long run (Kikuzawa 1995). Moreover, acclimation in *Fragaria vesca* has been shown to support physiological processes, most importantly photosynthesis in leaves (Chabot 1978).

Leaf senescence can be either induced by external factors or occur as a natural part of plant development. In both cases, there are always triggering factors. In perennial non-evergreen plants leaf senescence in cold climates is linked to changes in the environment, particularly temperature. Other possible factors include inadequate water or nutrient supply, changes in light regime, and pathogens. Because leaves contain many valuable substances, plants try to save as much of them as possible, and this recovery is a coordinated process. (Buchanan-Wollaston 1997) In the setup for this study, the major environmental factor connected to *Fragaria vesca* summer leaf senescence and winter leaf development was assumed to be low temperature.

1.3 The Role of Chlorophyll in Plants

Chlorophyll is the green pigment located in plant chloroplasts. The two types of chlorophyll in vascular plants, chlorophyll a and b, absorb red and blue wavelengths of light (Nishio 2000) and are essential components in the photosynthesis of higher plants. Chloroplasts, the sites of photosynthesis, are distributed unevenly in leaf cells because the cell vacuole takes up a significant portion of the cell content (Nishio 2000). Values of leaf chlorophyll content provide information concerning photosynthetic activity and thus also plant productivity (Cerovic et al. 2012). Chlorophyll is a valuable pigment that plants wish to retain, and thus leaf senescence is normally accompanied with mechanisms that allow plants to recover it. Chlorophyll loss can also be used as a measure when observing senescence in leaves (Noodén et al. 1997).

Photosynthesis as a process and the photosynthetic apparatus are subject to many challenges or even threats, which influence chlorophyll as well. These challenges are mostly related to the availability of light: light conditions for photosynthesis can be either optimal, saturating, which refers to more light energy reaching the leaf than is required for photosynthesis, or non-saturating, which means that light supply is insufficient for photosynthesis (Nishio 2000). Too much light energy may lead to over-excitation of the photosynthetic apparatus, which manifests itself as a drop in photosynthetic activity (Steyn et al. 2002) and various heat dissipation mechanisms such as evapotranspiration are then required (Nishio 2000). The temporarily decreased photosynthetic capacity is also known as photoinhibition (Long et al. 1994). When high light intensities are combined with stressful environmental factors, e.g. low temperatures and inadequate water supply, photoinhibitory damage to photosynthetic apparatus may occur (Hoch et al. 2001). The reason for this damage lies in reactive oxygen species, which chloroplasts create in suboptimal conditions (Gould 2004). Photobleaching in turn is the loss of fluorescence in photosynthetic pigments (Porret & Rabinowich 1937).

Both immature and senescing leaves suffer more readily from photoinhibition and photobleaching (Hoch et al. 2001, Krause et al. 1995). Evidently, photoinhibition and photobleaching have both negative effects on chlorophyll function in photosynthesis, as does insufficient light supply. Plants can adjust to varying light conditions both externally and internally. Externally, plants can alter light absorbance by leaf movements,

for example (Steyn et al. 2002). In addition to mechanisms such as evapotranspiration, plants can internally respond to high light levels both with the help of chlorophyll and secondary compounds in their leaves. These two groups of pigments act in different ways. Chlorophyll responds to changes in the light spectrum and adjusts light capture accordingly (Gould 2004). Secondary compounds offer tools for many stresses encountered by plants, including light, and these tools also include non-photosynthetic pigments such as anthocyanins (Wink 2010).

1.4 Secondary Compounds: Flavonoids, Flavonols and Anthocyanins

Plants have an abundance of different secondary compounds that are diverse both in their structure and in how they function. Common features to these substances include energetically costly synthesis, high concentration in storage, often long-distance transport and metabolic recycling. They are not vital to photosynthesis or other such profound plant processes, but have many important roles nevertheless. These include, for example, providing defense against plant pathogens, against other plants in competition and protection against physical stresses or UV light (Wink 2010). Cold temperatures, for example, are considered to be a physical stress to plants. The amount and type of secondary compounds in plants may express genetic variation (Kroymann 2011).

Plant secondary compounds include substances such as alkaloids, terpenoids, tannins and flavonoids (Kroymann 2011). In terms of chemistry, flavonoids are characterized by the presence of two benzene rings (Taylor & Grotewold 2005). In plants flavonoids have been connected to the polar transport of growth and regulatory hormone auxin, UV protection (Winkel-Shirley 2002), and they have also been shown to act in reducing the toxic effects of aluminum (Kidd et al. 2001). Moreover, different plants use flavonoids in different ways (Taylor & Grotewold 2005), and also control flavonoid genes in a different fashion (Winkel-Shirley 2002). Flavonoids can be divided into two major groups: non-pigmented and pigmented. Flavonols are a type of non-pigmented flavonoids, and the pigmented group consists of anthocyanins. According to Winkel-Shirley (2002), flavonols are significant among flavonoids due to their long history in the plant kingdom, wide distribution, and their biological activity.

Anthocyanins are a group of water-soluble pigments derived from flavonoids that can be seen as deep shades of red, purple and blue in plant tissues. These permanent or temporarily occurring compounds are present in many flowers, fruits and berries, but also often in plant leaves. Their appearance can be linked to environmental factors including cold temperatures, and they appear to have a connection to plant adaptation. (Chalker-Scott 1999) Being water-soluble, they are located in cell vacuoles (Landi et al. 2015) and their synthesis is thought to be light-dependent (Mol et al. 1996, Steyn et al. 2002). Anthocyanins are partly responsible for the fall-colors that people mostly connect to tree leaves, but which are also present in herbaceous plant leaves in the field layer, e.g. in *Vaccinium myrtillus* that also has anthocyanins in its berries.

Cold temperatures, either suddenly or as slower reductions promote anthocyanin synthesis and anthocyanins are often linked to acclimation (Christie et al. 1994). Nevertheless, the significance of anthocyanins in non-reproductive tissues is still not entirely clear. Anthocyanins have been connected to many environmental stress factors, including drought, UV-B radiation, pathogens or herbivores, and heavy metals (Gould 2004). Their transient nature and the large number of triggering factors behind their accumulation have made it difficult to specify their functions (Steyn et al. 2002). An important role for anthocyanins is to participate in plant photoprotection. Studies have shown that photo-oxidative damage to senescing leaf cells is reduced by the accumulation of anthocyanins (Hoch et al. 2001). Moreover, nutrient recovery is thought to benefit from photoprotection provided by anthocyanins (Hoch et al. 2001, 2003, Feild et al. 2001). Anthocyanins absorb the excess light instead of chlorophyll b and thus protect this valuable pigment (Gould 2004). This chain of events has also been referred to as the 'resorption protection hypothesis', which assumes that senescence turns leaves more vulnerable to photoinhibition and photoinhibition disrupts nutrient recovery (Hoch et al. 2003).

It is not only excessive visible light that anthocyanins shield plants from. UV-B radiation has the ability to damage photosystem II (PSII) in the photosynthetic apparatus of the chloroplasts (Teramura & Sullivan 1994). Research has shown that anthocyanins also absorb UV-B radiation, which protects plants form the harmful effects it can cause (Steyn et al. 2002). However, UV-B protection is connected to flavonoids in general, and research has led scientists to believe that UV-B protection is not a primary function for anthocyanins due to the location of these pigments in leaf internal layers (Gould 2004). Steyn et al. (2002) have suggested that the incidence of anthocyanins in the presence of UV-radiation is connected simply to controlling the effects of visible light on the photosynthetic apparatus.

Even though anthocyanin production may be beneficial for plant adaptation, it is not without its costs. The production of anthocyanins is expensive in terms of plant metabolism as well as transport (Gould 2004) and they may also interfere with photosynthesis (Chalker-Scott 1999). According to Nishio (2000), photosynthesis is less efficient when non-photosynthetic pigments capture light. However, the local distribution of anthocyanins in the leaf, namely whether in one or several layers in the epidermis, in mesophyll or in both, has a direct effect on how much less light is captured by chlorophyll. The costs of production may be justified because anthocyanins act in defense to changing conditions, either in development or in the environment, and usually anthocyanins are soon replaced by more permanent changes in plant metabolism. Moreover, the costs are more likely lower than damages that might occur without anthocyanins or the cost of other protective mechanisms. (Steyn et al. 2002) Thus, anthocyanins often act as first aid to sudden changes, while the plant develops more permanent responses.

2. Project Description and Hypotheses

This master's thesis focuses on ecophysiology in twelve different European woodland strawberry (*Fragaria vesca*) genotypes with measurements on summer and winter leaf size, on their chlorophyll, flavonol and anthocyanin content as well as with observations on their individual stolon production under controlled growing conditions and at different temperature treatments in a greenhouse. The principal aim of this study was to find out how lowering temperatures affect summer and winter leaf size development, stolon development, and leaf chlorophyll and secondary compound content in both winter and summer leaves of different *Fragaria vesca* genotypes. In connection to stolon development, one of the goals was to observe whether the potential termination of stolon production coincides with summer leaf senescence and winter leaf formation.

The work presented here continues previous research focusing on *Fragaria vesca* winter and summer leaf characteristics (Åström et al. 2015) and the photosynthetic activity of summer and winter leaves in different *Fragaria vesca* genotypes (Still 2019). A major difference is that these previous studies have been done in field conditions with fluctuating temperatures, weather conditions, exposure to frost, snow and several other naturally occurring environmental factors. Moreover, previous studies have not covered such an extensive range of *Fragaria vesca* genotypes.

By observing summer and winter leaf development, stolon development, and leaf chlorophyll and secondary compound content in both winter and summer leaves of different *Fragaria vesca* genotypes, it is possible to reveal possible connections between cold temperatures and winter leaf development. In a previous study in field conditions with ten *Fragaria vesca* genotypes from different countries, all the genotypes produced winter leaves (Still 2019). Therefore, the hypothesis in this study was that all genotypes would produce winter leaves too. The underlying assumption behind the setup was that cold temperature would trigger and affect winter leaf development, and that there would be differences both between the genotypes and between cold and warmer conditions. Therefore, the all-encompassing null hypothesis is that all the genotypes act in the same fashion within the same temperature, but also at different temperatures.

With such extensive study material, there is room for several comparisons. Firstly, the responses of different genotypes under the same conditions can be compared to each other. Secondly, it is possible to look at how different conditions affect a single genotype, e.g. whether there are differences in how a certain genotype develops in warmer and colder temperatures. Thirdly, if winter leaf development occurs, it is possible to compare the winter and summer leaves of different genotypes in the same conditions, and finally, to also make comparisons of winter and summer leaves within a single genotype in different conditions. However, for the purposes of this study, the research questions and hypotheses (H_x) were limited to the following:

Summer and winter leaf size

- Are the summer/winter leaves of different genotypes different in size?
  - ➔ H_1: The summer/winter leaves of different genotypes are different in size.
- Does temperature and/or genotype affect the size of winter leaves?
  - ➔ H_2: Temperature and/or genotype do influence winter leaf size.

Stolon development

- Do different genotypes at the same temperature stop producing stolons at the same time?
  - ➔ H_3: Different genotypes at the same temperature stop stolon production at different times.
- Do individual genotypes stop producing stolons at the same time despite the temperature?
  - ➔ H_4: Individual genotypes stop producing stolons at different times at different temperatures.
- Does temperature and/or genotype affect the number of developing stolons?
  - ➔ H_5: Temperature and/or genotype influence the number of developing stolons.

Chlorophyll and secondary compound content

- Are there differences in summer leaf chlorophyll/flavonol/anthocyanin content at different temperatures or between genotypes?
  - ➔ H_6: The summer leaf chlorophyll/flavonol/anthocyanin content differs according to temperature both within and between genotypes.
- Are there differences in winter leaf chlorophyll/flavonol/anthocyanin content at different temperatures or between genotypes?
  - ➔ H_7: The summer leaf chlorophyll/flavonol/anthocyanin content differs according to temperature both within and between genotypes.

3. Materials and Methods

3.1 *Fragaria vesca* Genotypes

The material for the study presented in this thesis consisted of twelve European *Fragaria vesca* genotypes that have originally been collected from wild in five European countries: Finland, Norway, Germany, Italy and Spain. These genotypes (marked NOR5, NOR3, FIN53, FIN51, FIN50, GER12, GER4, IT20, IT14, ES18, ES12, ES2) have been gathered from natural habitats in these countries and then kept for research purposes in Finland on University of Helsinki Viikki campus by Timo Hytönen's Strawberry Research Group, Department of Agricultural Sciences, Faculty of Agriculture and Forestry and Viikki Plant Science Centre (VIPS).

The *Fragaria vesca* genotypes used in this study, their places of origin and the coordinates of original collection sites are listed below in Table 1. Moreover, the growth altitude of these locations is also listed. A map of the original locations of each genotype except FIN53 and ES12 is presented in the master's thesis of Sonja Still (Still 2019). For the purposes of this study, this selection of twelve *Fragaria vesca* genotypes offered a comprehensive source of information concerning this species in terms of latitudes, altitudes and differences within and between countries as well as climatic conditions.

Table 1: *The origin of Fragaria vesca genotypes used in the study. The genotypes are listed from north to south in a latitudinal order. The table presents the coordinates (coordinate system: WGS84) and the altitude of each genotype's original collection site. Altitudes for all genotypes except three marked with an asterisk in the table had been determined in conjunction with collecting the plants. The missing altitudes were determined with the help of ESRI ArcGIS-online maps (2020).*

Genotype	Place of Origin	N coordinate	E coordinate	Altitude
NOR5	Norway: Alta, Rafsbotn, Rishaugen	70.0226	23.55951	<50 m
NOR3	Norway: Alta, Leirbukta	69.93955	23.09714	<50 m
FIN53	Finland: Lohja	60.2076	23.8066	<45 m*
FIN50	Finland: Raasepori, Karjaa	60.1061	23.6782	25 m
FIN51	Finland: Raasepori	60.0673	23.2879	20 m
GER4	Germany: River Ilm valley, Garden of J.W. Goethe, Weimar	50.9847	11.3225	250 m*
GER12	Germany: Vorvogelsberg, Ulrichstein, Bröllwiesenwald, Hessen	50.65205	9.162438	310 m*
IT20	Italy: Montagnaga	46.1279	11,2462	880 m
IT14	Italy: Barcesino, Cima Sat	45.87086	10,7848	660 m
ES12	Spain: Astúrias (Oviedo), La Almuña	43.5339	-6,5271	200 m
ES18	Spain: Huesca, entre Hecho y Ansó	41.5212	0.3531	1000 m
ES2	Spain: Jaén, (Las Acebeas)	37.7796	-3.7849	1320 m

The study presented in this master's thesis is also part of a larger research project concerning *Fragaria vesca* genotypes and their ecophysiology. Therefore, when data was collected for this thesis, it was also collected for a doctoral dissertation by Sonja Still and for the Plant Ecophysiology and Climate Change Group (PECC) at University of Helsinki, Faculty of Biological and Environmental Sciences, Finland. This additional data included fluorescence measurements, observations on the number of leaves and the timing and development of winter leaves in *Fragaria vesca*. Since these measurements were completed for other research projects, this thesis does not present or discuss this additional data in detail.

3.2 Experimental Setup

The plants for this study were acquired as stolon clones (ramets) from the original genotypes kept on Viikki campus. From each genotype twenty clones plus two extra individuals were collected. An exception was one genotype, ES18, from which exactly twenty individuals were acquired because the mother plant had not produced any more. These clones were then planted into small pots (7x7 cm) in Kekkilä's peat-based Professional Substrate at the beginning of September 2019, covered with a transparent plastic sheet and placed in a greenhouse. Twenty plants per genotype was considered to be a large enough group for making generalizations, even when the groups were eventually divided into two temperature treatments. The small pots were chosen for primary planting so that the *Fragaria vesca* would root better, and the purpose of the plastic sheet was also to improve rooting and growth. The plastic cover, however, caused yellowing in some of the *Fragaria vesca* leaves (Figure 1). Nevertheless, the yellowing soon disappeared after the plastic sheet was removed before the first measurements started three weeks after the clones had been planted.

At the time of planting, each individual plant in a pot was labeled with a tag stating the code for the genotype, e.g. NOR3, and the number (1-20) of the plant (Figure 1). The extra individuals had no number, only the genotype code. The tag was placed at the far side of each pot so that it would interfere with plant growth as little as possible. The purpose of the tags was to allow each measurement to be linked to each individual plant. Extra individuals were planted so that there was emergency supply in case anything went wrong with the original twenty individuals during the course of the experiment. This proved to be useful since in fact, one of the Italian individuals, IT20 1, had to be replaced after a few weeks because it died for unknown reasons.

In the beginning all the plants were kept at +16°C (Figure 3). Because the first pots were relatively small and the individual plants grew fairly rapidly, after six weeks of starting the experiment the *Fragaria vesca* were planted into larger pots (13 cm in diameter) with the same Kekkilä soil that was used during the first planting (Figure 1). Three weeks later half of the plants from each genotype were transferred to +11°C (Figure 3). The other half, control group, remained at +16°C throughout the experiment. On this same occasion the placement of the pots was also randomized so that different genotypes were mixed on the greenhouse tables. By this time the growth of plant individual ES18 20 had been so slow and the plant was still so tiny that it was removed from the group. Since there were no extra individuals of genotype ES18, the cold treatment for ES18 consisted of only nine individuals instead of ten. After ten individuals from each genotype had been kept at +16°C and +11°C for six weeks, the temperature in the cooler room was lowered further to +6°C, and the strawberries were kept in this cooler temperature for four weeks, until the end of the study period (Figure 3).

The three temperatures, +16°C, +11°C and +6°C, were chosen based on previous knowledge on *Fragaria vesca* growth and greenhouse capacity regarding temperature regulation. The highest temperature, +16°C, was the starting point because it was a generally used temperature for cultivating plants at the greenhouse and based on previous studies *Fragaria vesca* were known to be still actively growing in this temperature. The lowest temperature, +6°C, was chosen simply because it was the lowest temperature that could be produced in the Viikki greenhouses. The middle point, +11°C, was handily between the two extremes and it was used in order to provide a gradual decrease of temperature to the plants instead of an abrupt ten-degree drop that could have caused various shock effects and damage in the plants. Moreover, a gradual reduction mimicked natural conditions in the sense that normally temperatures in the fall decrease in such a fashion that plants have time to create responses to these changes by acclimation. The lighting in the rooms was provided by high pressure sodium (HPS) lamps that were set to be on for eighteen hours before the plants were divided into two temperature treatments. After this, the day length was set to twelve hours.

Figure 1: *Fragaria vesca two weeks after planting (A), after planting to larger pots (B) and rubber band markings (C). A: Initial planting to small pots. Yellowing caused by the plastic sheet cover can be seen at the edges of some leaves. The individual orange tags can be seen clearly at the sides of the pots. B: Fragaria vesca arrangement on the greenhouse table and the overall mass of all the 240 + 20 extra plants before they were divided to two rooms. C: New leaves were marked with colored rubber bands, first ones with red.*

Because one of the goals of this study was to find out more about summer and winter leaf size in *Fragaria vesca*, the leaves of each *Fragaria vesca* plant were surveyed throughout the experiment. In order to be able to know when possible winter leaves would start developing, starting from 5 October 2019 until 20 December 2019 the leaves growing from the main rosette of each plant were marked every two weeks with rubber bands that were different in color according to the marking day. The rubber band was placed around the petiole of the youngest fully-grown leaf that had appeared from the rosette (Figure 1). With the information provided by these markings the starting point for collecting winter leaf data was then determined. These markings caused no harm to the leaves or the plants because the rubber band was easy to place around the leaf petiole.

3.3 Stolon Development Observations

The experiment period started on 21 September 2019, and during the first two weeks the work focused on counting and removing all stolons that were longer than 5 cm in each *Fragaria vesca* plant. Shorter ones were allowed to stay on until they had grown longer. This process continued every week throughout the 15-week study period, from 21 September to 27 December 2019 (Figure 3).

The stolons were counted and removed each week for several reasons. First, it helped to distinguish when each genotype would stop producing them. This information could then be linked to the timing of summer leaf senescence and winter leaf production. Second, counting and removing the stolons helped to prevent the plants from spreading too much, and in the worst case mixing with each other. It also stopped the greenhouse table becoming overcrowded as the plants grew. However, removing the stolons also stopped the *Fragaria vesca* from using a great portion of energy on producing them or clones on them. From beginning on, in addition to removing stolons after they had been calculated, all side branches or axillary shoots were also removed. This was done because allowing them to grow would have enlarged the rosette considerably as well as made it difficult to count the stolons correctly, and to know which leaves were emerging from the original shoot.

3.4 Summer and Winter Leaf Size Measurements

Previous studies have shown that *Fragaria vesca* summer and winter leaves have morphological differences that are reflected in the leaf size (Åström et al. 2015). Thus, summer and winter leaves were measured also in this study. The purpose was to find out whether there were differences in leaf size between genotypes in the same temperature and within genotypes between the cool and warm temperature treatment. In October, before any temperature treatments had been started and before the summer leaves had shown signs of senescence, the petiole length and the length and width of the middle leaflet were measured from

the first marked summer leaf in each plant, which in fact was one of the last summer leaves of the growing season and also the leaf used for summer leaf measurements in this study. This meant that at the time of leaf size measurements two weeks had passed since the leaves had been marked, and they were fully grown.

The same measurement procedure was then repeated towards the end of the experiment on 7 December 2019 to what were assumed to be winter leaves. These winter leaves were also the ones used in the Dualex measurements. The winter leaf size data was collected from plants in two different temperature treatments, which also allowed comparisons between treatments.

3.5 Dualex Measurements

In addition to counting and eliminating stolons and measuring summer and winter leaf size, the experiment also included leaf chlorophyll and secondary compound content measurements with a FORCE-A Dualex Scientific+ meter (Dynamax Inc., Houston, Texas, USA). The FORCE-A Dualex Scientific+ meter applied to the measurements provides information on leaf chlorophyll, flavonol, anthocyanin and nitrogen content, but the Nitrogen Balance Index provided by the meter is actually a combination of the chlorophyll and flavonol values (Cerovic et al. 2012).

The Dualex meter determines leaf chlorophyll content by using two light impulses, near-infrared and red. Red is partly absorbed by the leaf because it causes chlorophyll fluorescence, and the chlorophyll value is a difference in transmission of the two wavelengths. In a similar fashion, Dualex determines leaf flavonol and anthocyanin content by using two light impulses, UV- and red light for flavonols and green and red light for anthocyanins. The epidermis emits the red wavelength and absorbs the UV/green light. The fluorescence of these two light impulses is compared to each other, which allows to determine the epidermal absorbance that corresponds to flavonol and anthocyanin content. (FORCE-A 2011).

The measurement probe of Dualex Scientific+ consists of two round clips (Figure 2 A). The measurements are done by opening the gap between the clips and carefully placing a leaf between them. In a few seconds the meter beeps as a signal for completed measurement and the values of each parameter become visible on the small display. If necessary, it is possible to delete the individual measurement immediately after completing it and redo it.

The meter is an easy tool to operate, small in size and light in weight. Moreover, it is indifferent to temperature or light conditions and provides a simultaneous measurement result for all the parameters. It has been found to be an accurate meter for these measurement purposes. The unit of measurement for chlorophyll content with this device is $\mu g/cm^2$ as a factory calibration, but the flavonol and anthocyanin values have no unit, since they are measured in a different fashion by the meter. (Cerovic et al. 2012)

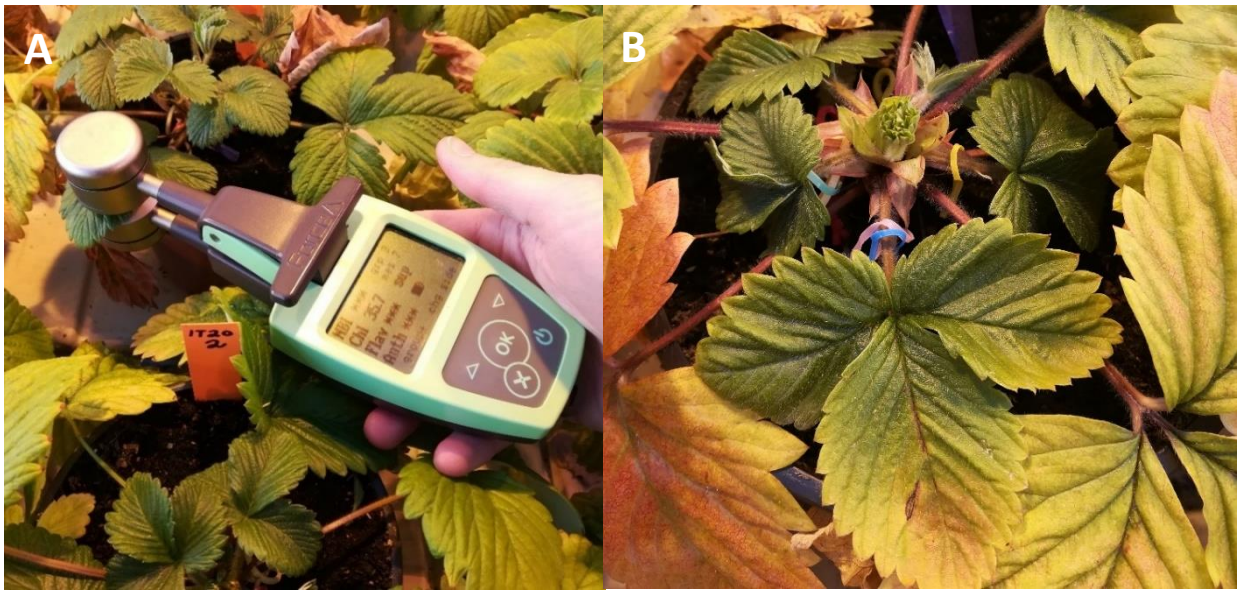


Figure 2: *Dualex meter and its form of operation (A) and winter leaf necrosis (B). A: The measurements were always conducted by placing the Dualex clips at the side of the middle leaflet of the marked leaf. B: A marked and measured winter leaf showing signs of chlorosis/necrosis on the last day of the measurements.*

Benefits of the Dualex meter include the fact that it does not harm the leaves. However, in this study the winter leaves of some genotypes, e.g. NOR3 and NOR5, were so tiny that measuring them proved to be very challenging. Moreover, because the leaves were not entirely smooth, it was sometimes difficult to obtain a valid measurement. This meant that the meter had to be adjusted and the measurement repeated, which required recurrent touching. Thus, it is possible that this and the repeated measurements overall caused some thigmomorphogenesis (Jaffe & Forbes 1993) in the plant leaves. Over the course of weeks this could be seen as chlorotic or necrotic spots in the areas where the Dualex meter had been placed (Figure 2 B).

The first Dualex measurements were started during the third week of the control period when all the individual plants were still kept in the same room, at the same temperature of +16°C. At this point all the genotypes had grown sufficiently for successful measurements. Before this, the leaves of some genotypes, e.g. ES18, were too small for using Dualex. Completing measurements on the plants before they were divided into two rooms provided data that could then be used as a reference to verify that possible later occurring differences would not be caused by initial differences of plant individuals within genotypes.

After the first five weeks in the same room, half of the plants were moved into the cooler room and the Dualex measurements continued on a weekly basis for the summer leaves. When what appeared to be winter leaves had started developing, the Dualex measurements were started on them as well. The summer leaf Dualex measurements were continued for as long as the leaves started senescing and became too dry for measurements to be completed. However, it was not always clear when they had dried up and died fully, so they were measured to be on the safe side. If the leaf had fully senesced, the values obtained from these measurements were not accurate, so they had to be removed from the data before analysis.

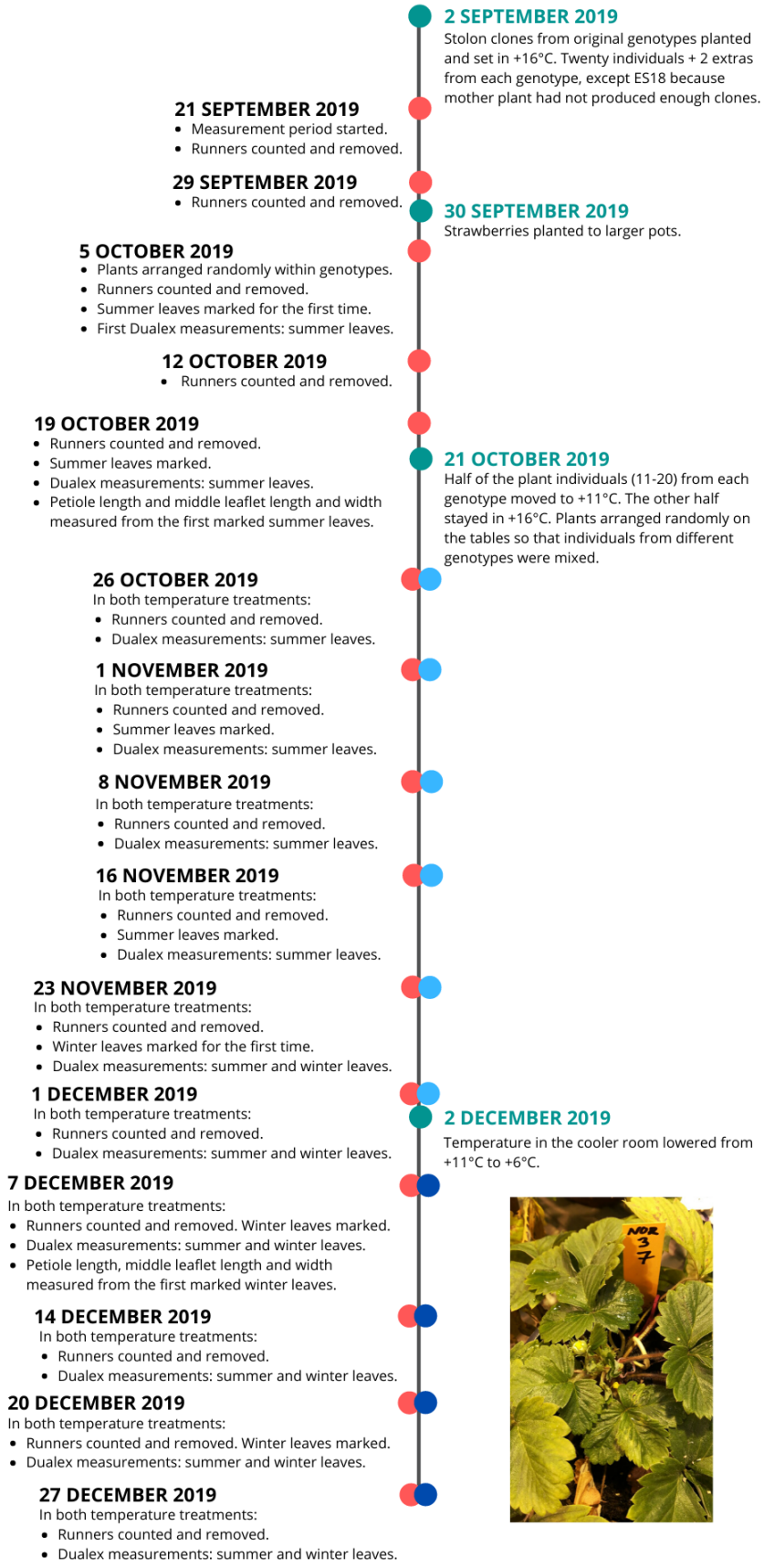


Figure 3: *Experiment setup and measurement timeline. Measurement dates on the left are marked in black. Transition dates are presented in turquoise color on the right side of the timeline. Temperature treatments are marked with red (+16°C), light blue (+11°C) and dark blue (+6°C) circles on the timeline.*

3.6 Data Analysis

The collected data was first organized and viewed in Microsoft Office Excel. Graphs and other visualizations were also created with Excel. After this, the data was analyzed statistically with the IBM SPSS Statistics 26 (IBM, USA) statistical software. Statistical analyses were completed for winter leaf size data, stolon data and Dualex chlorophyll, flavonol and anthocyanin content data. The nitrogen content Dualex data was excluded from the analyses because it combines the existing values of chlorophyll and flavonol levels and does not provide new information as such. Similarly, no statistical tests were done with the summer leaf size data because the measurements had been completed while the all plants were still at the same temperature. Thus, no comparisons could be made.

The winter leaf size data was analyzed with a two-way Anova including a Tukey post hoc test and with genotype and temperature as independent variables. Stolon data from the ten weeks when the strawberries were kept in two different rooms was analyzed with a two-way repeated measures Anova including a Tukey post hoc test with genotype and temperature as dependent variables. The data from the first five weeks could not be added to this same analysis due to different group sizes (20 instead of 10). The same type of two-way repeated measures Anova test was also done with the summer and winter leaf chlorophyll, flavonol and anthocyanin content data, where the data from two temperature treatments of summer or winter leaves was combined for the analyses.

4. Results

4.1 Leaf Size

In terms of leaf size, the twelve genotypes chosen for this study had visible phenotypic differences to begin with. Both the Norwegian genotypes, NOR3 and NOR5, had relatively small leaves and short petioles. In contrast, the Italian genotypes, IT20 and IT14, had very large summer leaves and IT14 also long petioles. Genotype ES18 could be described as fragile, with fewer leaves, small leaf size and long petioles. The Norwegian and Finnish genotypes had very small winter leaves and towards the end of the experiment they started producing buds in both rooms and commenced flowering in the warm room. In these genotypes there were open flowers during the last day of measurements at the end of December (Figure 4 A). One of the German genotypes produced extra leaflets that were attached to its petiole (Figure 4 B). The visually observed differences in summer and winter leaf size can be verified with the help of statistical analyses and plots created from the measurement data.

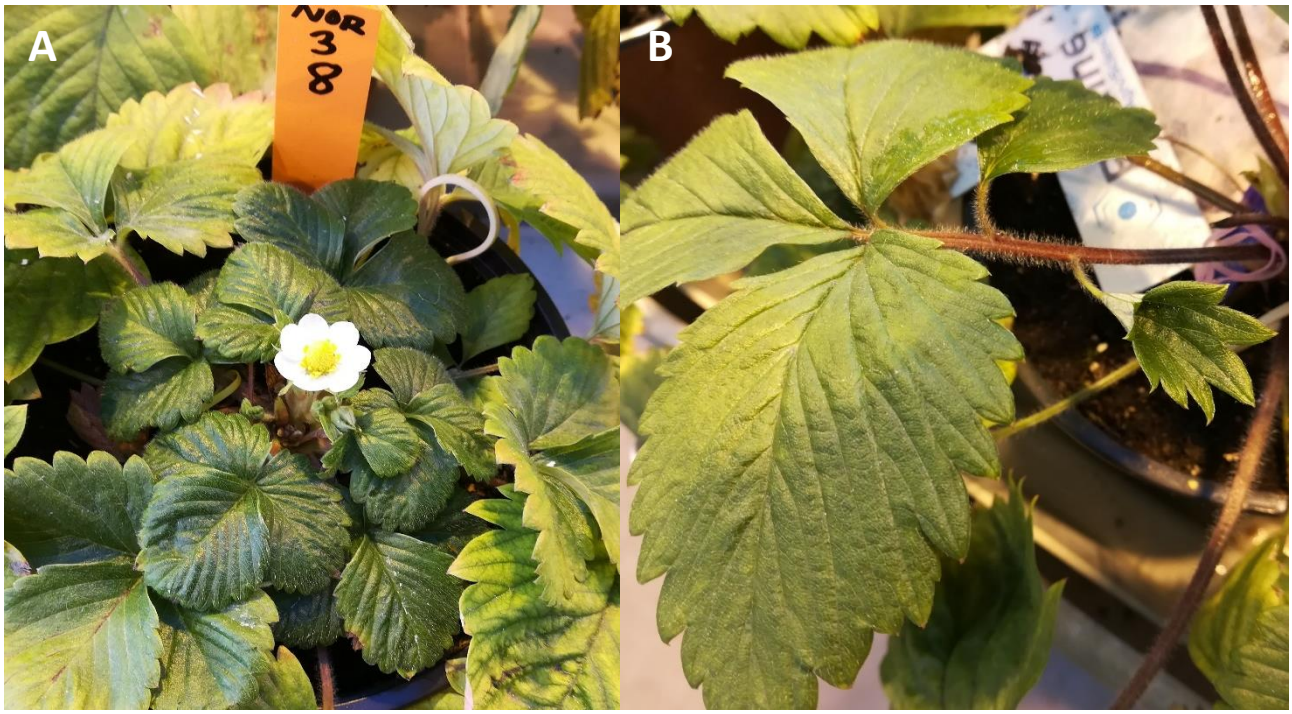


Figure 4: *Genotype NOR3 in flower (A) and genotype GER12 extra leaflets (B). The image on the left (A) shows a flower in one of the warmer room NOR3 plants on the last day of the measurements, 27 December 2019. The image on the right (B) shows the extra leaflets that genotype GER12 produced on its petiole.*

The statistical two-way Anova tests for middle leaflet width, middle leaflet length and petiole length in winter leaves show that for all these parameters both genotype (middle leaflet width: p=0.000, F=24.893, df=11; middle leaflet length: p=0.000, F=50.528, df=11; petiole length: p=0.000, F=72.343, df=11) and temperature (middle leaflet width: p=0.000, F=53.45, df=1; middle leaflet length: p=0.002, F=9.781, df=1; petiole length: p=0.000, F=353.971, df=1) have an effect on the measured values. Furthermore, in all cases the interaction of genotype and temperature was also statistically significant (middle leaflet width: p=0.001, F=2.894, df=11; middle leaflet length: p=0.004, F=2.625, df=11; petiole length: p=0.000, F=6.088, df=11).

The post hoc Tukey tests for winter leaf size measurements reveal that in middle leaflet width genotypes NOR5 in the cold treatment and ES12 in the warm treatment differed significantly from other genotypes, treatments included. A similar difference in middle leaflet length was found in genotypes NOR3 in the cold treatment, NOR5 in the cold treatment and GER12 in the warm treatment. The post hoc Tukey tests for winter leaf petiole length show that genotypes ES18 in the warm treatment, ES12 in the warm treatment, NOR3 in cold treatment and NOR5 in cold treatment differed significantly from other genotypes. Based on these tests, in all size measurements the winter leaves of genotype NOR5 in the cold treatment differ significantly from the size of other genotype winter leaves in both warm and cold treatments.

In the summer leaves, genotype GER4 had the greatest variability in middle leaflet width (Figure 5 A). It also had the widest individual leaves. Both Norwegian genotypes had relatively similar summer leaflet width

values and genotype FIN53 resembled them. Genotype ES18 had the smallest summer leaf middle leaflet width, although with variation. In what were assumed to be winter leaves, the leaflet widths in the warmer room (Figure 5 C) were generally slightly greater compared to the colder room (Figure 5 B). Genotype ES18 had larger winter leaf width in the warmer room (Figure 5 C), and these values were also greater than the summer leaf values. Similarly as with summer leaves, genotype GER4 had the greatest variety in winter leaf width and also largest individual values in both temperature treatments.

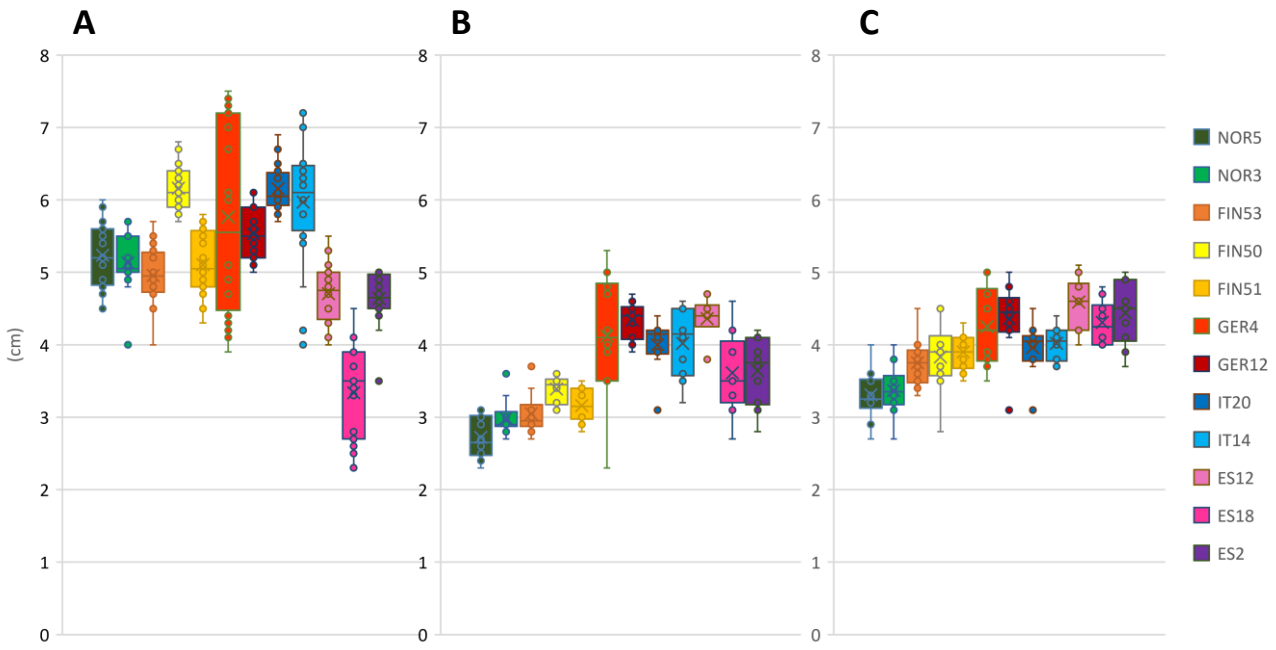


Figure 5: *Fragaria vesca middle leaflet width averages, quantiles and outliers of each genotype summer and winter leaves. A: Summer leaf middle leaflet width. B: Winter leaf middle leaflet width, cold room (+6°C). C: Winter leaf middle leaflet width, warm room (+16°C). Summer leaves: n=20, winter leaves: n=10, except for ES18 n=9.*

In genotype ES12 the middle leaflet width average was almost the same in both summer and winter leaves (Figure 5), but in this genotype there was little variety within the winter leaf width in the cold room (Figure 5 B). The Norwegian and Finnish genotypes had the smallest winter leaf width of all the genotypes despite temperature differences between the two rooms. Surprisingly, genotype FIN50 had a large summer leaf width, an average of 6 cm together with the Italian genotypes, whereas the other Finnish and Norwegian genotypes had averages closer to 5 cm in their summer leaf widths (Figure 5 A).

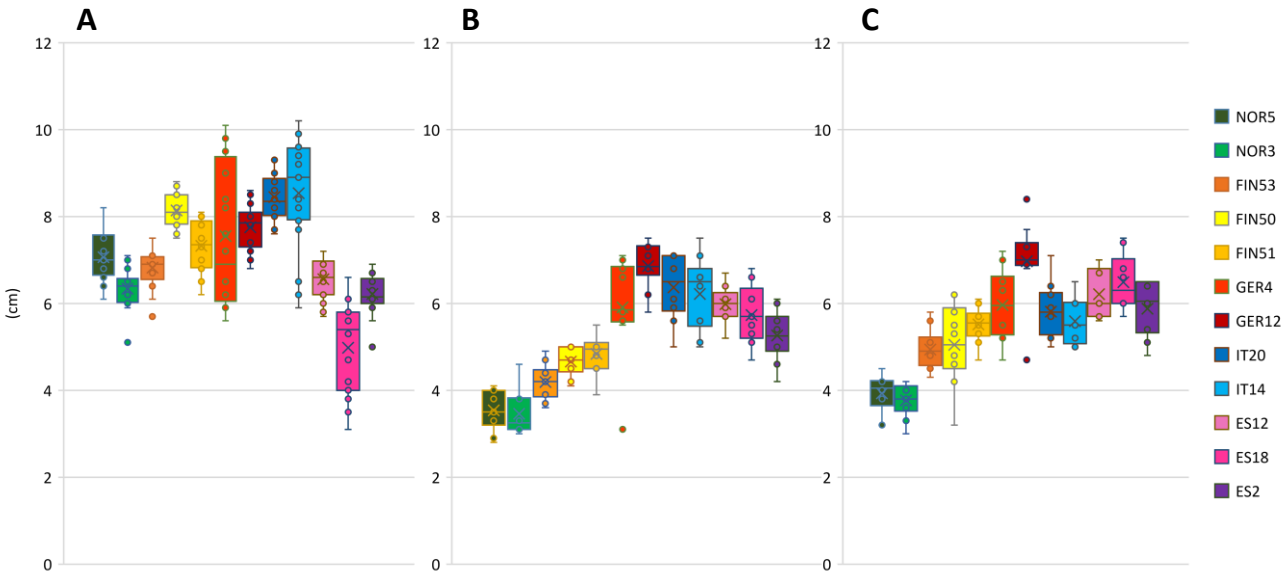


Figure 6: *The middle leaflet length averages, quantiles and outliers of each genotype summer and winter leaves. A: Summer leaf middle leaflet length. B: Winter leaf middle leaflet length, cold room (+6°C). C: Winter leaf middle leaflet length, warm room (+16°C). Summer leaves: n=20, winter leaves: n=10, except for ES18 n=9.*

In summer leaf middle leaflet length (Figure 6 A), the genotypes followed a relatively similar pattern compared to middle leaflet width, but IT20 had the greatest length and as a close second, GER4 had large diversity in the length measures. Genotype ES18 had the shortest summer leaf middle leaflet length and FIN50 had greater length than its Finnish and Norwegian counterparts.

In winter leaf middle leaflet length genotype GER12 instead of GER4 had the largest values in both rooms (Figure 6 B-C), but also an outlier in the warmer room (Figure 6 C). Comparably with middle leaflet width, the winter leaflet length of Norwegian and Finnish genotypes in both temperature treatments was smaller than those of other genotypes. Moreover, the values for winter middle leaflet length for the Italian genotypes were smaller than the Spanish ones in the warmer room as was the case with middle leaflet width as well (Figure 6 C). Overall, the winter leaf length in all genotypes in both temperature treatments was smaller than summer leaf middle leaflet length, except in genotype ES18. In ES18 the winter leaf middle leaflet length in both temperature treatments was greater than in summer leaves.

In summer leaf petiole length (Figure 7 A) genotype IT14 rose clearly above the other genotypes, but the other Italian genotype, IT20 had much shorter petioles. The genotypes NOR5 and NOR3 had the shortest summer leaf petioles in general. FIN50 had relatively long petioles too, coming close to IT14. The summer leaf petioles (Figure 7 A) were clearly longer than the winter leaf petioles (Figure 7 B-C). In summer leaves the range was approximately 6-11 cm and in winter leaves 2-7 cm in the cold room (Figure 7 B) and 3-9 cm in the warm room (Figure 7 C).

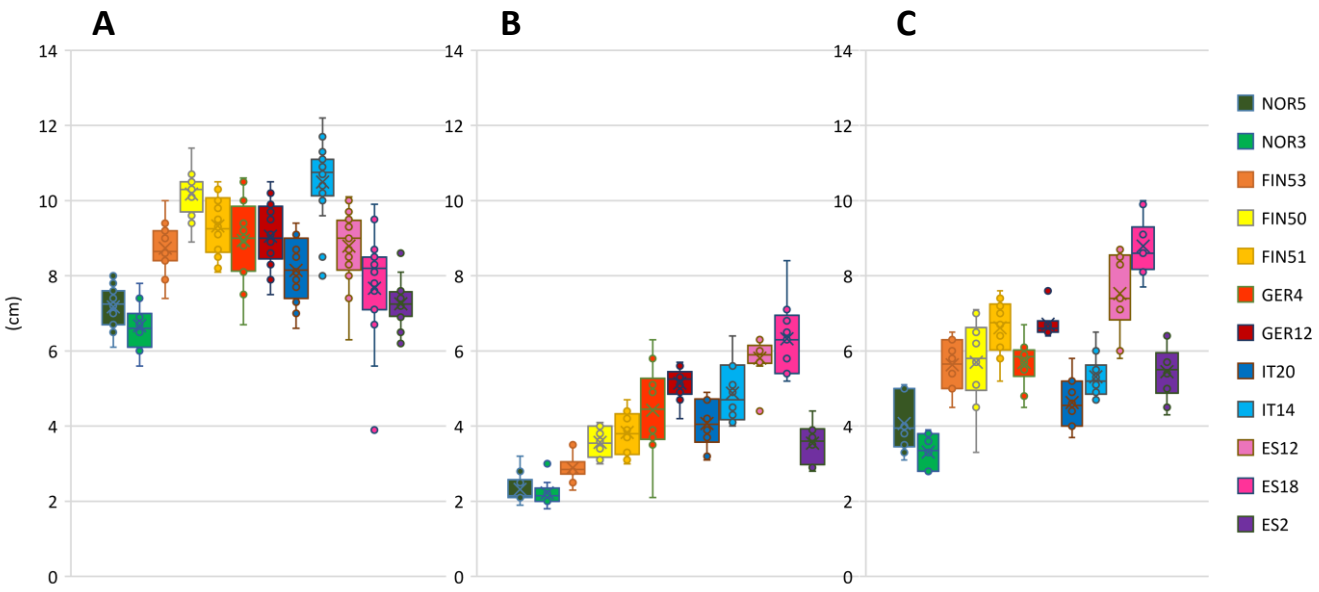


Figure 7: *Petiole length averages, quantiles and outliers of each Fragaria vesca genotype summer and winter leaves. A: Summer leaf petiole length. B: Winter leaf petiole length, cold room (+6°C). C: Winter leaf petiole length, warm room (+16°C). Summer leaves: n=20, winter leaves: n=10, except for ES18 n=9.*

The winter leaf petiole length values (Figure 7 B-C) break any patterns that could be seen with middle leaflet length and width. The Spanish genotypes ES12 and ES18 suddenly had the largest values and genotype ES2 had the lowest Spanish values in both temperature treatments. Moreover, in the warmer room (Figure 7 C) the Finnish genotypes were no longer the smallest together with the Norwegians, but their petioles were longer than the Italian ones. In petiole length the German genotypes dropped to middle range in both summer and winter leaves and in both winter leaf temperature treatments. The Norwegian genotypes, NOR5 and NOR3 had the shortest winter leaf petioles in the cold room (Figure 7 B) with an average close to 2 cm.

Summer leaf senescence was first observed in genotype FIN51 in the warm room at the beginning of November. This was followed by genotypes FIN53, then ES2, both in the warm room. In mid-November, the first individuals in the cooler room were expressing leaf senescence in genotype ES2. In general, leaf senescence was more rapid in the warmer room and less fall colors were produced (Figures 8-9). In the cooler room the formation of first winter leaves was observed in early November and by mid-November most genotypes appeared to have produced winter leaves. In the warmer room some genotypes had produced new leaves that were smaller than previous summer leaves, so they were assumed to be winter leaves.

Figure 8: *Fragaria vesca plants in the warmer room at the greenhouse at the end of December 2019. The plants have developed small winter leaves, but the summer leaves have mostly died without producing fall colors.*

Figure 9: *Fragaria vesca plants in the colder room at the greenhouse at the end of December 2019. The summer leaves have developed fall colors and new, smaller winter leaves have appeared.*

4.2 Stolon Development

During the fifteen-week experiment period the stolons growing on each plant individual were counted every week. The genotype differences in stolon production were clear. Some genotypes produced several stolons throughout the experiment, and some stopped producing them as the experiment progressed. In the cooler room stolon production generally ceased earlier than in the warmer room (Figures 10-11).

The statistical two-way repeated measures test on stolon measurements shows that the number of stolons is affected by both genotype (p=0.000, F=107.507, df=11) and temperature (p=0.000, F=658.568, df=1), and the interaction of these factors is also significant (p=0.000, F=23.133, df=11). Thus, the tendency to produce stolons in general is genotype-specific, but also temperature-dependent. In post hoc Tukey tests genotype ES2 in the cool treatment, ES12 in the cool treatment, GER12 in the warm treatment, NOR5 in the warm treatment and ES12 in the warm treatment differed significantly from the other genotypes in both treatments.

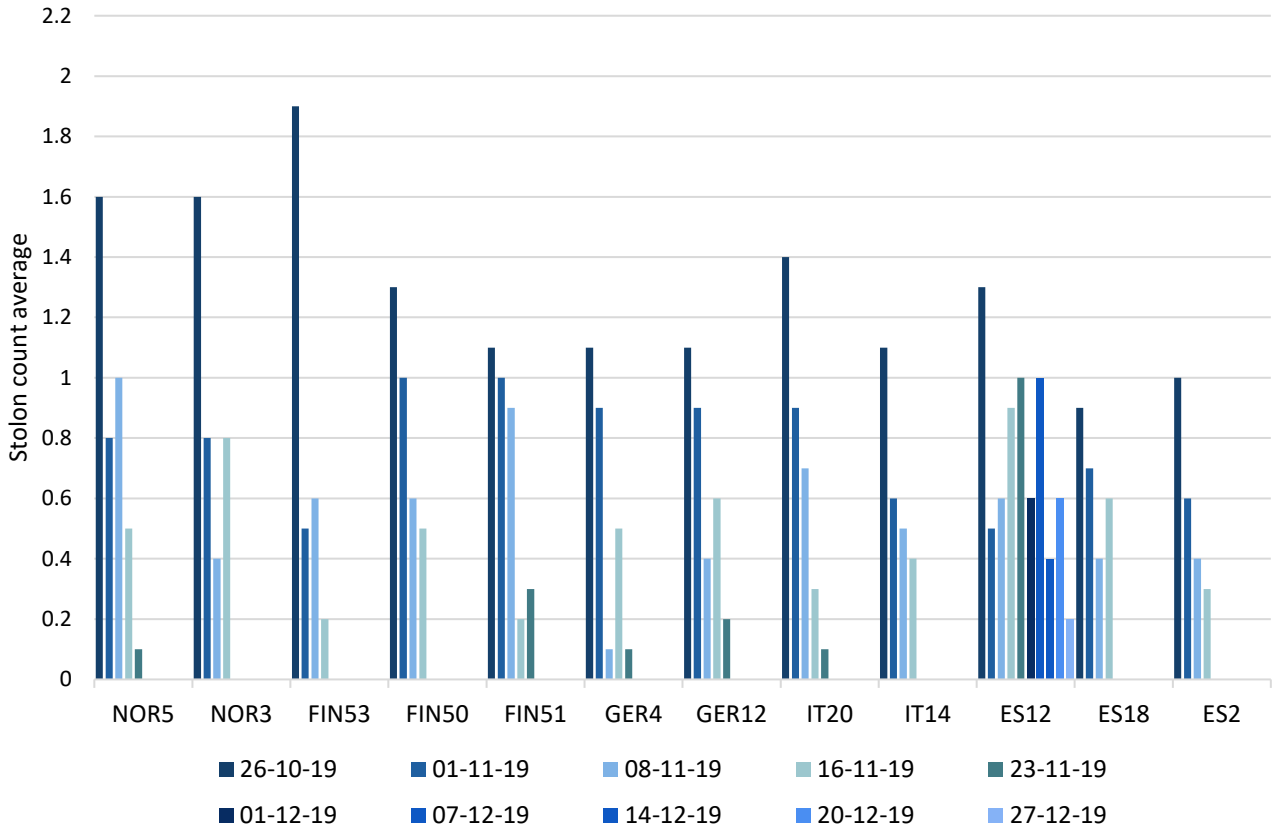


Figure 10: *The average number of stolons in twelve Fragaria vesca genotypes in the cold room. The stolons were counted every week from 26 October 2019 to 27 December 2019. N=10, except for ES18 n=9.*

Figure 10 above shows that most *Fragaria vesca* genotypes in the cooler room produced their last stolons already during November even though production in the warmer room (Figure 11) still continued. The last stolons in genotypes NOR3, FIN53, FIN50, IT14, ES18 and ES2 were counted and removed on 16 November 2019, only four weeks after they had been transferred to a lower temperature. Genotypes NOR5, FIN51,

GER4, GER12 and IT20 followed this trend during the following week, on 23 November 2019. It is worth noting that the stolon production of these genotypes stopped already at the temperature of +11°C, before the coldest treatment. In the cold room genotype ES12 was the only one to produce stolons until the last week of the experiment, which means that in this genotype the stolon production continued even throughout the lowest +6°C temperature treatment. According to Figure 10, during the first week after being transferred to the cooler room from the warm room in genotypes NOR5, NOR3 and FIN53 the average number of stolons was clearly greater than during the three following weeks. Thus, their stolon production declined sharply and rapidly. In the other genotypes the decline was less drastic.

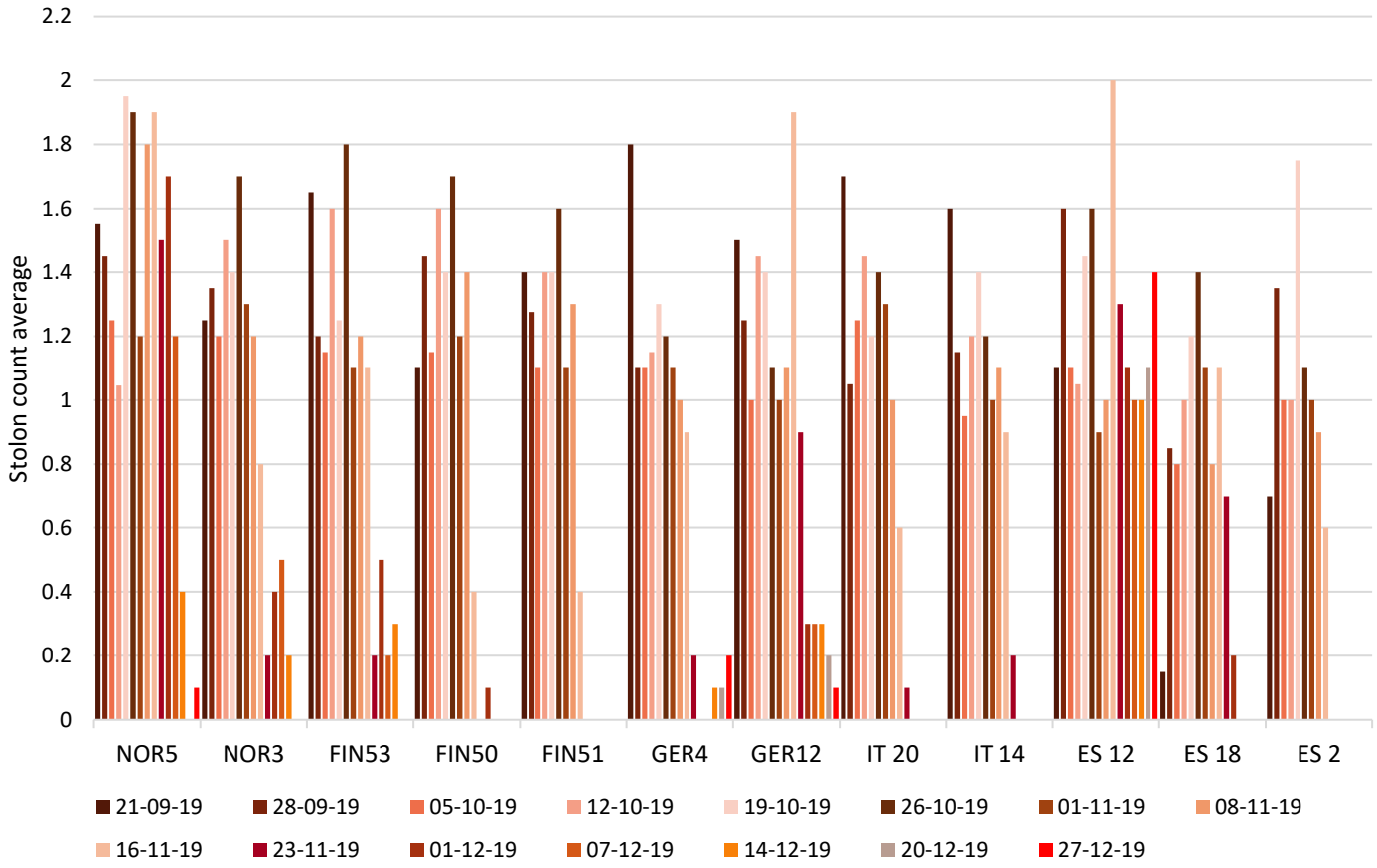


Figure 11: *The average number of stolons in twelve Fragaria vesca genotypes in the warm room. The stolons were counted during each week of the experiment period from 21 September 2019 to 27 December 2019. Before 26 October 2019 n=20, from 26 October 2019 onwards n=10.*

In the warm room both genotypes ES12 and GER12 were active stolon producers throughout the experiment, but in ES12 the production was steady whereas in GER12 the production did decline towards the end of December (Figure 11). Genotypes FIN51 and ES2 were the first ones stop producing stolons in the warm room. The last stolons in these genotypes were counted on 16 November 2019, which for FIN51 was a week earlier than in the same genotype in the cold room. Genotypes IT20 and IT14 had their last stolons on 23 November 2019 and FIN50 and ES18 the following week. Stolon development in genotypes NOR5, FIN50 and

GER4 stopped for a moment, but was activated again. Genotypes NOR3 and FIN53 produced stolons until mid-December. In general, the stolon production of most genotypes in the warm room either decreased or stopped after mid-November, except in ES12. However, compared to the cooler room (Figure 10), the average number of stolons starting from 26 October 2019 was slightly greater.

A surprising phenomenon related to stolon production occurred during the last few weeks of the experiment. As already mentioned, the plants also grew axillary shoots to the side of the original shoot. Some genotypes produced several of them, some a few, and the rest none at all. At the beginning the axillary shoots always emerged at some distance below the apex and they were easy to remove. However, after approximately two thirds of the experiment had passed, in some genotypes (e.g. NOR5, NOR3, FIN51) the axillary shoots were very close to the stem apex and appeared embedded in the crown, which made it seem as if the plant had more than one developing leaf of the same age (Figure 12). These side branches were very difficult to remove without harming the plant itself. In the end, it was also difficult to determine which new leaf belonged to the original growth.

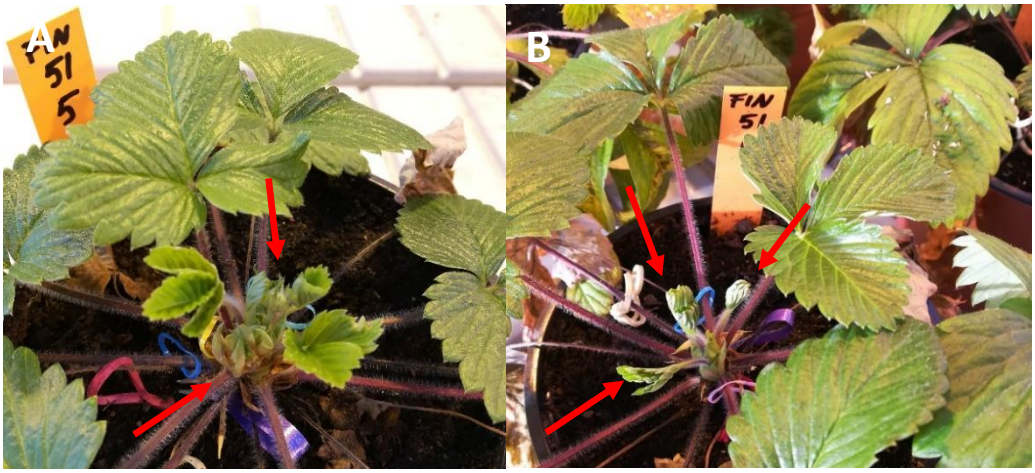


Figure 12: *Axillary shoots growing next to the apical meristem (A and B).*

4.3 Chlorophyll Content in *Fragaria vesca* Leaves

The two-way repeated measures analysis of variance statistical test for summer leaf chlorophyll show that both genotype (p=0.000, F= 17.599, df=11), temperature (p=0.008, F=7.095, df=1) and their interaction (p=0.000, F=6.549, df=11) have a significant effect on summer leaf chlorophyll content. The same type of statistical analysis for winter leaf chlorophyll content produced slightly different values. In between-subjects effects tests of genotype (p=0.000, F=10.488, df=11), temperature (p=0.037, F=4.399, df=1) and their interaction (p=0.037, F=2.744, df=11) the results are all significant, but to a slightly lesser extent compared to summer leaves. Particularly the effect of temperature is less significant than in summer leaves.

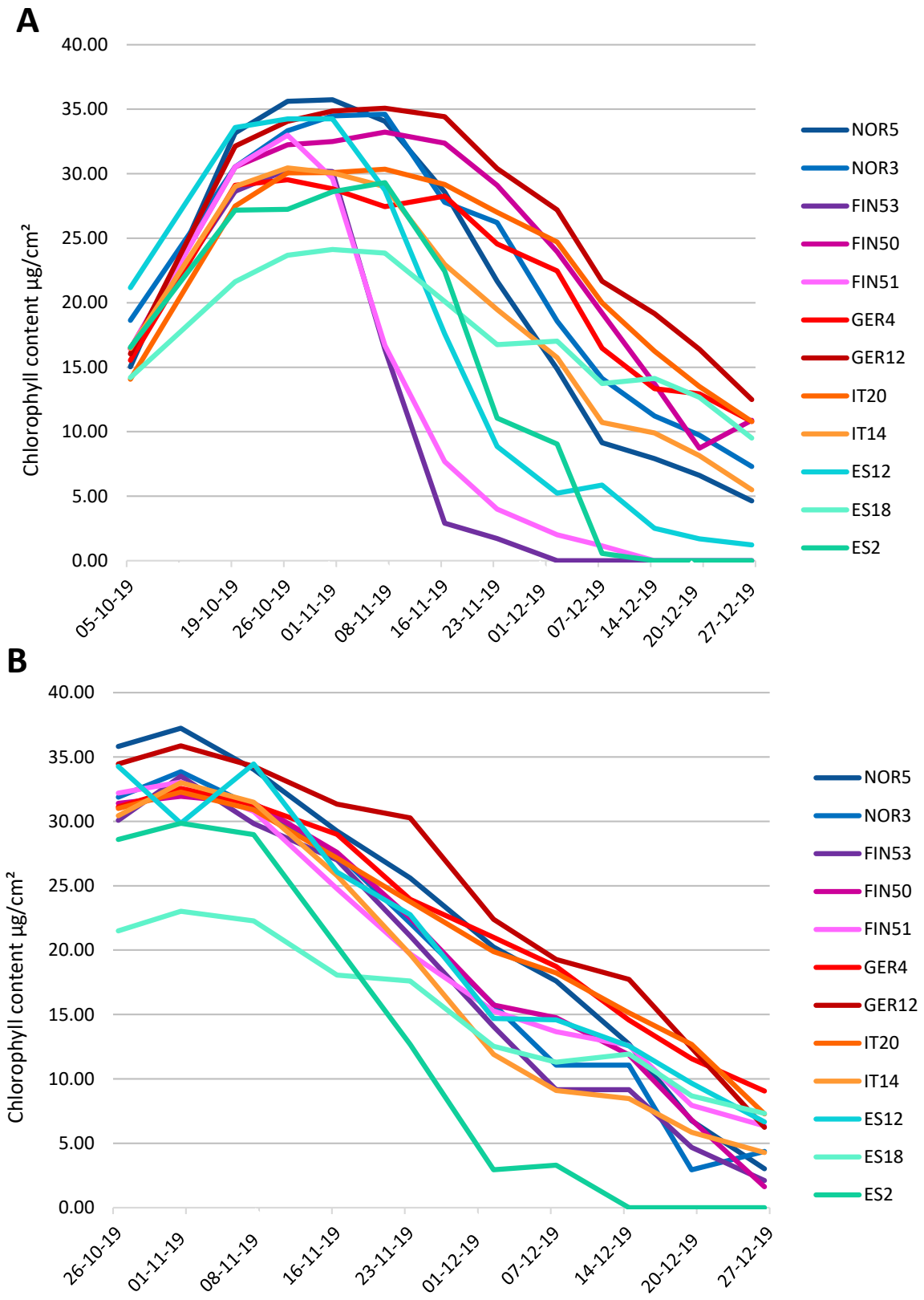
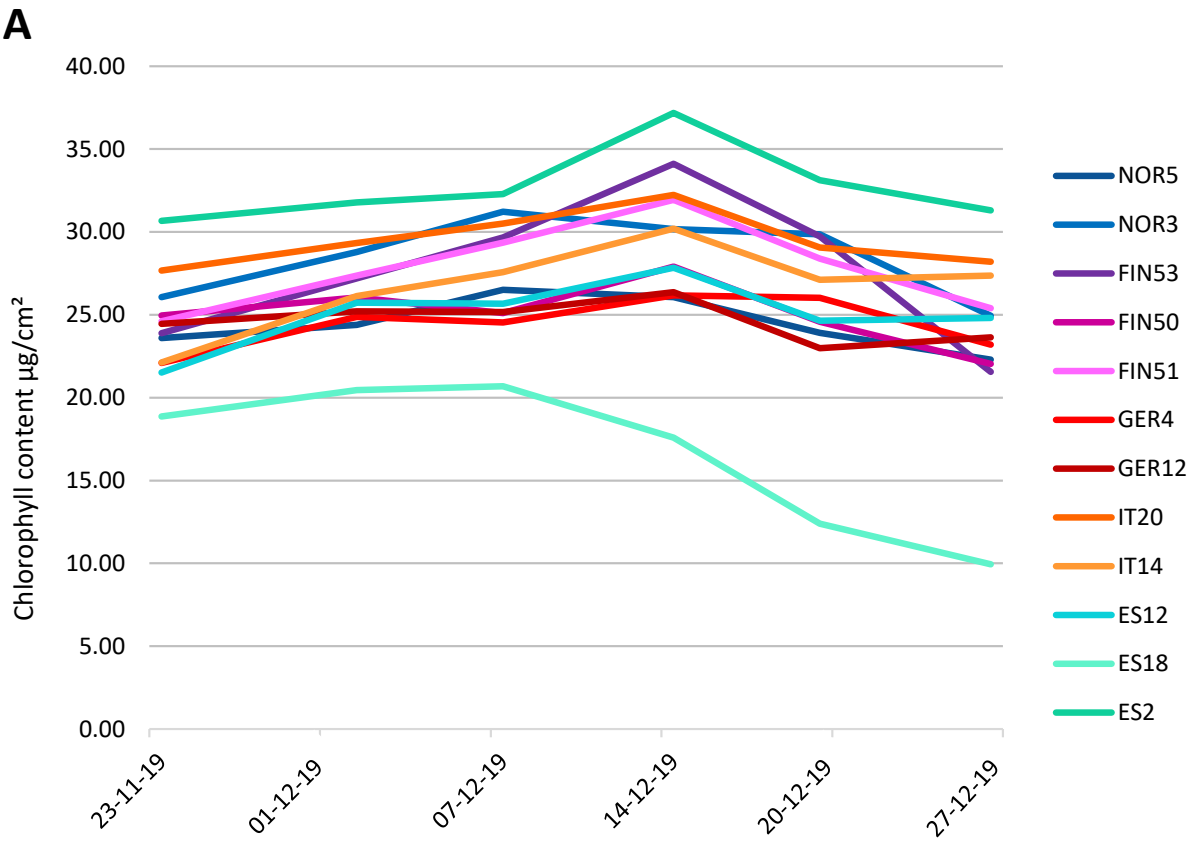


Figure 13: *The summer leaf chlorophyll content of twelve Fragaria vesca genotypes in two different temperature treatments. A: Summer leaf chlorophyll content in the warm room, measured from 5 October 2019 to 27 December 2019. B: Summer leaf chlorophyll content in the cold room, measured from 26 October 2019 to 27 December 2019.*

The chlorophyll content of summer leaves was measured twice from all strawberry individuals in the warm room before half of the individuals were transferred to the cold room. Figure 13 shows the summer leaf chlorophyll content in both warm (A) and cold (B) temperature treatments. In the warm room the chlorophyll content of all genotypes increased for the first few weeks and then started decreasing in the beginning of November (Figure 13 A). The chlorophyll content of genotypes FIN53 and FIN51 in the warmer room dropped quickly, with ES12 and ES2 following close behind them. The chlorophyll levels of FIN53, FIN51 and ES2 all reached zero by mid-December, which basically meant that all the measured leaves had fully senesced by then. These results are in line with the visual observations concerning leaf senescence.

Chlorophyll content in the ES2 summer leaves of plants in the cold room (Figure 13 B) dropped quickly and reached zero in mid-December. In all other genotypes the chlorophyll level decreased starting from the beginning of November, but the decrease was more gradual compared to genotype ES2 and also compared to the development in the warm room summer leaves. The chlorophyll level of genotype ES18 was lower to begin with: a little over 20 µg/cm^2 while the others started around the levels of 30-35 µg/cm^2, the same values in both treatments. Similarly as in the warm room, in the cold room the summer leaves of genotype GER12 had the highest chlorophyll levels throughout the experiment.



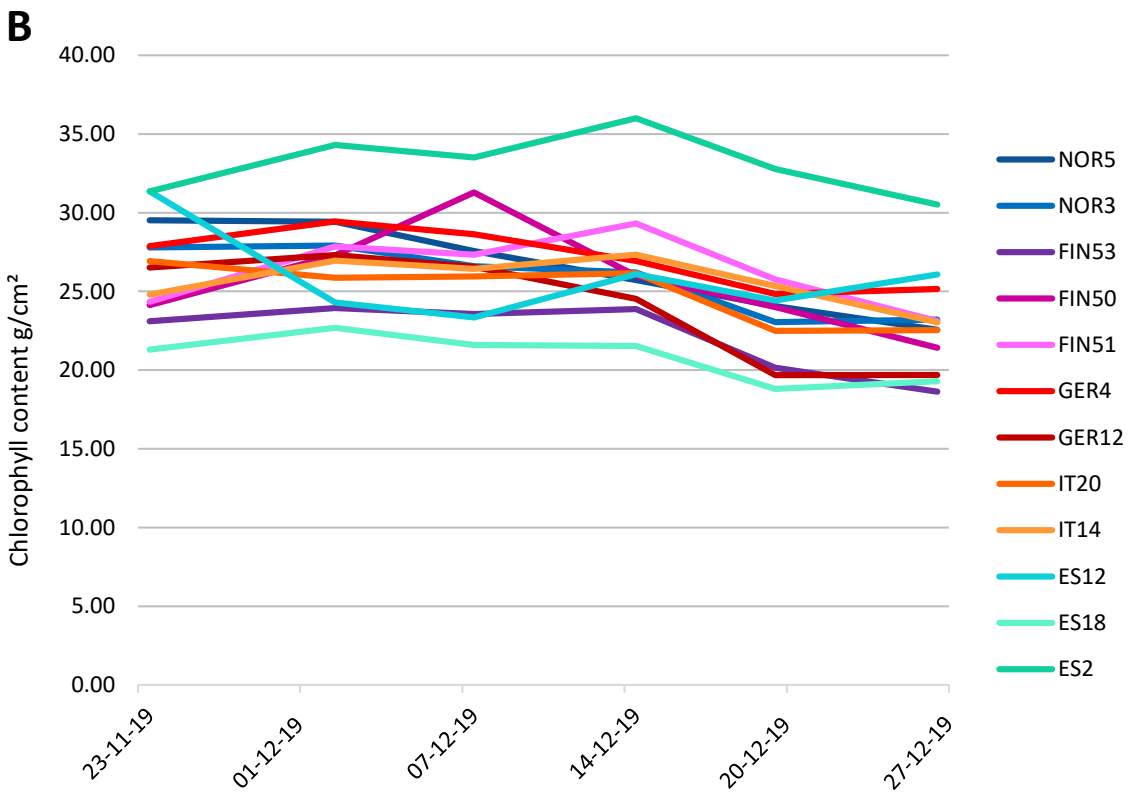


Figure 14: *The winter leaf chlorophyll content of twelve Fragaria vesca genotypes in two different temperature treatments. A: Winter leaf chlorophyll content in the warm room, measured from 23 November 2019 to 27 December 2019. B: Winter leaf chlorophyll content in the cold room, measured from 23 November 2019 to 27 December 2019.*

Figure 14 shows the chlorophyll content of *Fragaria vesca* winter leaves in both warm (A) and cold (B) treatments. The winter leaf chlorophyll level was the highest in genotype ES2, in both temperature treatments. In the warm room most genotypes had a small peak in winter leaf chlorophyll content on 14 December 2019 followed by a small drop the next week. Genotype ES18 in turn had the lowest winter leaf chlorophyll levels in both rooms, but in the warm room they were significantly lower than any of the others. The starting values of winter leaf chlorophyll (Figure 14 A-B) were greater than the summer leaf initial chlorophyll values (Figure 13 A-B).

4.4 Flavonols

According to the summer leaf statistical two-way repeated measures Anova test with temperature and genotype as fixed factors, both genotype ($p=0.000$, $F=12.999$, $df=11$) and temperature ($p=0.000$, $F= 108.618$, $df=1$) have a significant effect on summer leaf flavonol content. The interaction of these two factors ($p=0.000$, $F=8.14$, $df=11$) is also significant. In a similar fashion, the statistical two-way repeated measures Anova test for winter leaf flavonol content shows that genotype ($p=0.000$, $F=27.436$, $df=11$), temperature ($p=0.000$, $F= 682.67$, $df=1$) and their interaction ($p=0.000$, $F= 6.273$, $df=11$) have a significant effect on winter leaf flavonol content.

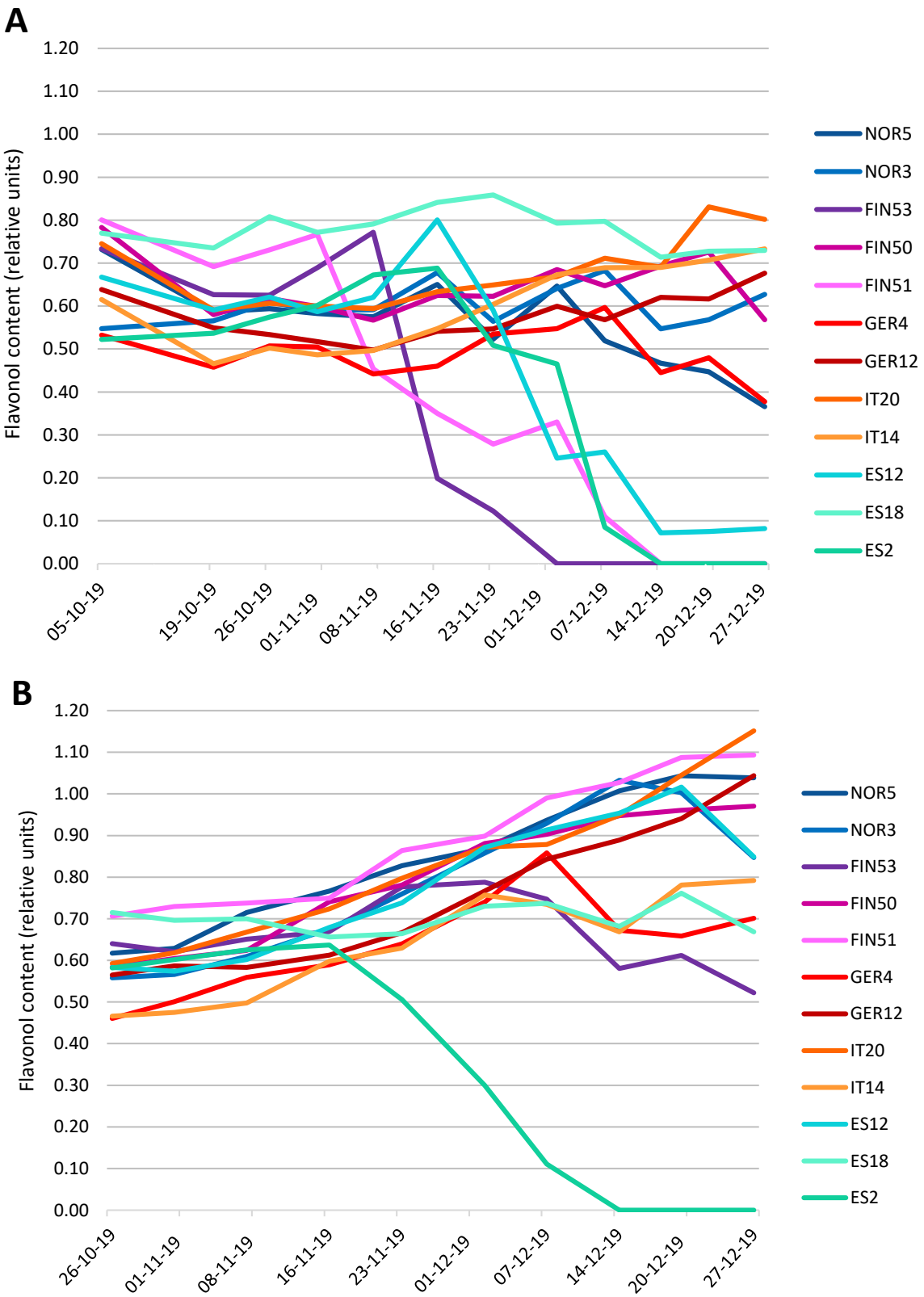
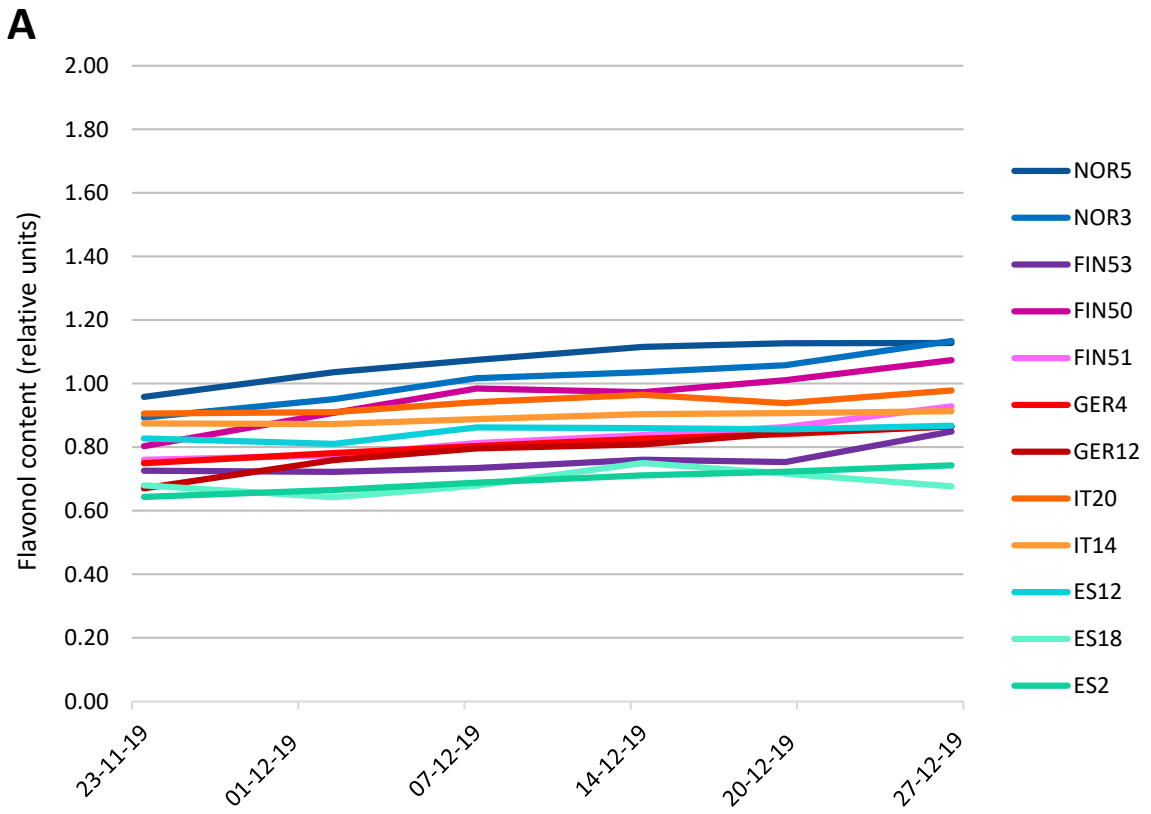


Figure 15: *The summer leaf flavonol content of twelve Fragaria vesca genotypes in two different temperature treatments. A: Summer leaf flavonol content in the warm room, measured from 5 October 2019 to 27 December 2019. B: Summer leaf flavonol content in the cold room, measured from 26 October 2019 to 27 December 2019.*

The starting point of flavonol content in summer leaves was relatively similar in the two temperature treatments to begin with, but differences soon emerged (Figure 15). The summer leaf flavonol content in the warm room shows fluctuation and differences between genotypes (Figure 15 A). The flavonol values of genotypes FIN53, FIN51, ES2 and ES12 began dropping in November. The process was the same in summer leaf chlorophyll content in the warm room (Figure 13 A). Genotype FIN53 was the first to reach zero, which meant that all its summer leaves had died. Genotypes FIN51 and ES2 also reached zero before the end of December. In genotypes NOR5, FIN50 and GER4 the flavonol content also began decreasing. Overall, ES18 had the highest summer leaf flavonol content in the warm room, although IT20 peaked in the end.

In the cold room (Figure 15 B), genotype ES2 had similar summer leaf flavonol values with others to begin with, but the flavonol content soon decreased and reached zero in mid-December. In genotypes NOR3, FIN53, GER4, ES12 and ES18 the flavonol content decreased during December. In genotypes NOR5, FIN50, FIN51, GER12 and IT20 the summer leaf flavonol content increased steadily throughout the measurement period in the cold room.



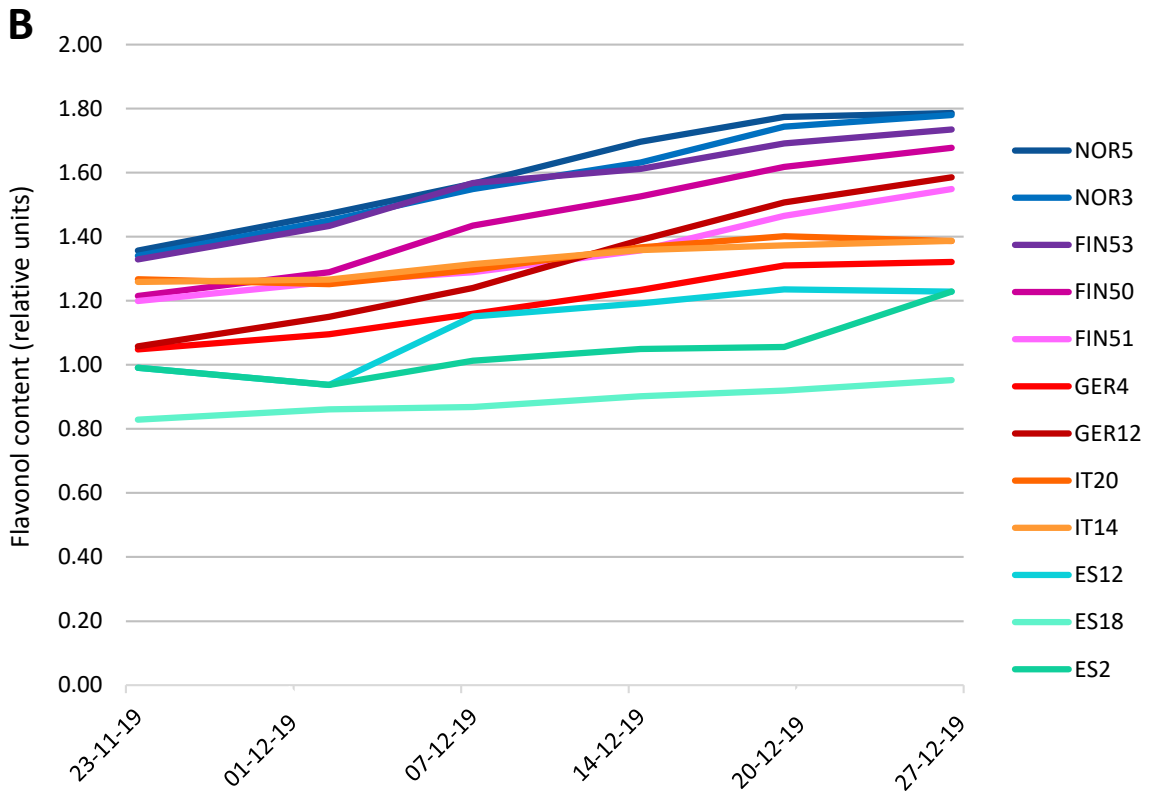


Figure 16: *The winter leaf flavonol content of twelve Fragaria vesca genotypes in two different temperature treatments. A: Winter leaf flavonol content in the warm room, measured from 23 November 2019 to 27 December 2019. B: Winter leaf flavonol content in the cold room, measured from 23 November 2019 to 27 December 2019.*

The winter leaf flavonol content presented in Figure 16 shows a steady pattern in both temperature treatments (A-B). However, the values in the cold room winter leaves (Figure 16 B) are generally higher and steadily increasing compared to the warm room (Figure 16 A). The graphs of different genotypes in the cold room show higher dispersal than the graphs in the warm room. In the warm room genotype NOR5 has the highest flavonol content with ES2 and ES18 having the lowest values and FIN53 close above them. In the cold room winter leaves genotypes NOR5, NOR3, FIN53 and FIN50 have the highest values and the Spanish genotypes the lowest. In both temperatures the values for the German and Italian genotypes are mixed.

4.5 Anthocyanins

According to the statistical two-way repeated measures Anova test for summer leaf anthocyanins with temperature and genotype as fixed factors, genotype (p=0.000, F=4.677, df=11), temperature (p=0.000, F=105.585, df=1) and their interaction (p=0.000, F=3.599, df=11) have a significant effect on summer leaf anthocyanin content. The same applies to winter leaf anthocyanins: statistical tests show that genotype (p=0.000, F=4.4, df=11), temperature (p=0.000, F=22.432, df=1) and their interaction (p=0.000, F=3.433, df=11) also have a significant effect on winter leaf anthocyanin content.

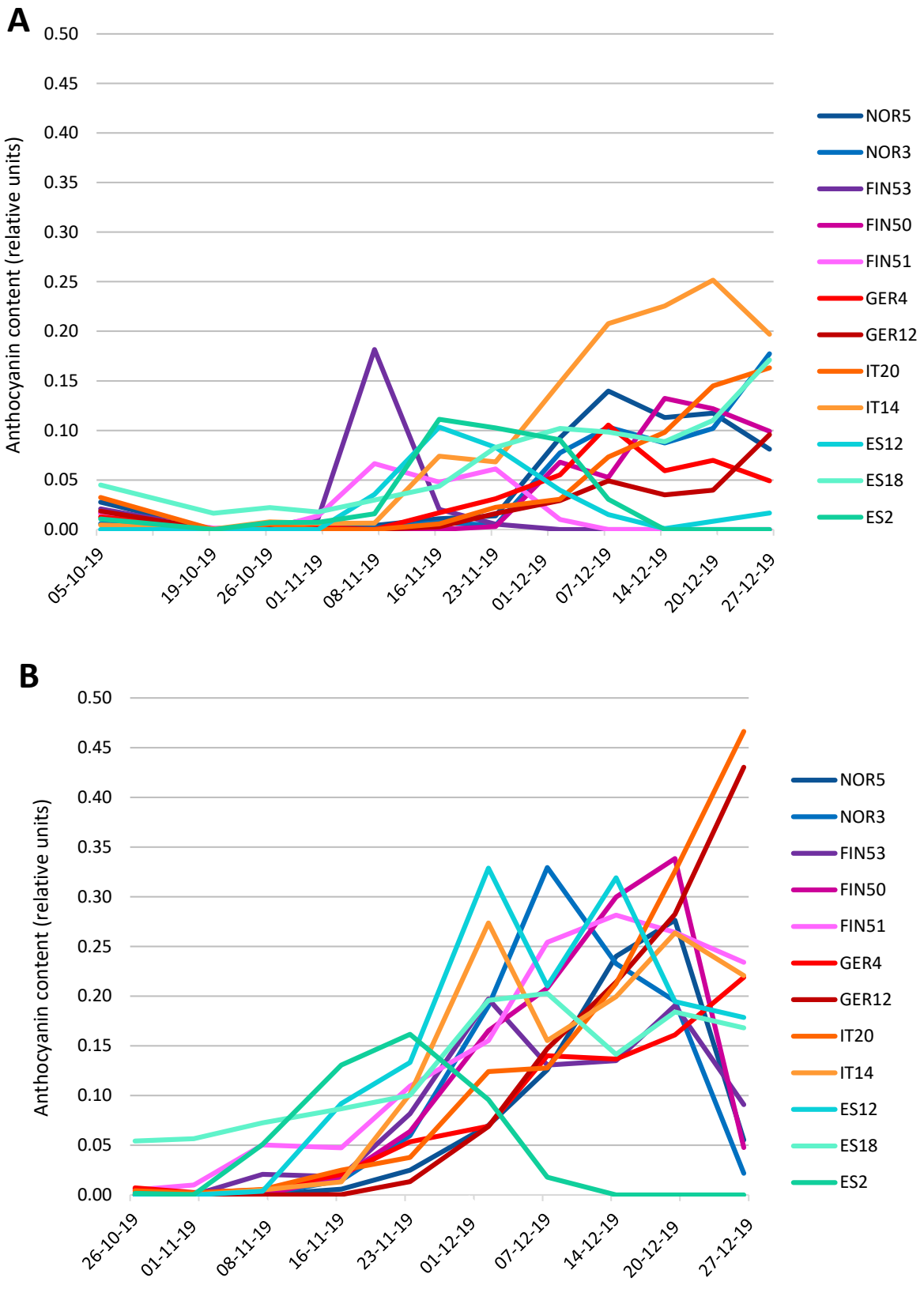


Figure 17: *The summer leaf anthocyanin content of twelve Fragaria vesca genotypes in two different temperature treatments. A: Summer leaf anthocyanin content in the warm room, measured from 5 October 2019 to 27 December 2019. B: Summer leaf anthocyanin content in the cold room, measured from 26 October 2019 to 27 December 2019.*

The anthocyanin content of summer leaves in both temperature treatments shows considerable fluctuation within and between genotypes (Figure 17 A-B). According to Figure 17 A, genotype FIN53 in the warm room had a sudden peak in anthocyanins at the beginning of November, but then declined sharply towards zero. The IT14 genotype had the highest anthocyanin content in the warm room, but the values were already declining during the last two measurements. Other genotypes with increasing values towards the end of December were NOR3, IT20 and ES18. Genotypes FIN51 and ES2 dropped to zero in December and genotype ES12 had decreasing values that reached almost zero and then slightly increased during the last two weeks of the experiment. The highest summer leaf anthocyanin value in the warm room was 0.25 by IT14.

In the colder room (Figure 17 B) the summer leaves of genotypes IT20 and GER12 stand out with their high and rapidly increasing anthocyanin values. In IT20 the average anthocyanin content during the last day of measurements was approximately 0.47. Genotypes IT14, ES12, NOR3, ES12, FIN51, NOR5 and FIN53 had peaks in their anthocyanin content one after the other from the end of November towards the end of December. Compared to the other genotypes in the cold room, the anthocyanin levels of ES2 summer leaves express a different pattern. This genotype had its highest anthocyanin values already on 23 November 2019, and then the values soon declined to zero, reflecting summer leaf senescence in this genotype.

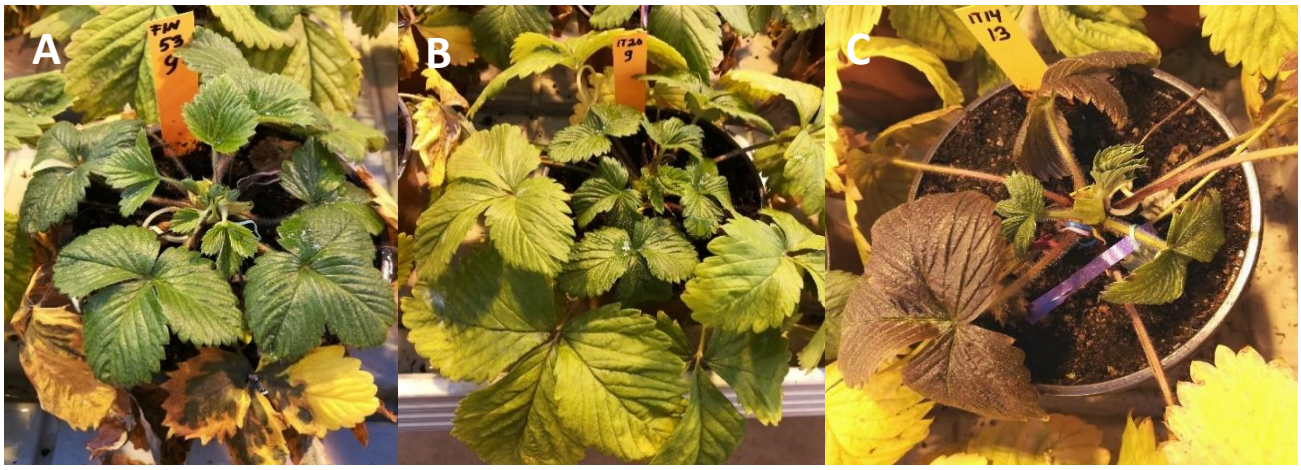


Figure 18: *Summer and winter leaves in both temperature treatments at the end of December. A: The summer leaves of FIN53 in the warm room have turned yellow and dried. Smaller, dark green winter leaves have developed. B: The Italian genotype IT20 in the warm room has also developed winter leaves, but the larger summer leaves are still alive around them. C: In the cold room the summer leaves of genotype IT14 have changed their color considerably due to anthocyanin production and smaller, dark green winter leaves are growing from the rosette.*

In winter leaves (Figure 19 A-B) the anthocyanin values are very different compared to the summer leaves. In the warm room (Figure 19 A) genotype ES18 stands apart from the other genotypes with its early-induced, rapidly increasing anthocyanin values that reach an average of approximately 0.19 during the last day of the measurements. All the other genotypes in the warm room remained under 0.05 in their average winter leaf anthocyanin content throughout the 6-week measurement period. Genotypes ES12 and ES2 produced hardly any anthocyanins at all in the warm room.

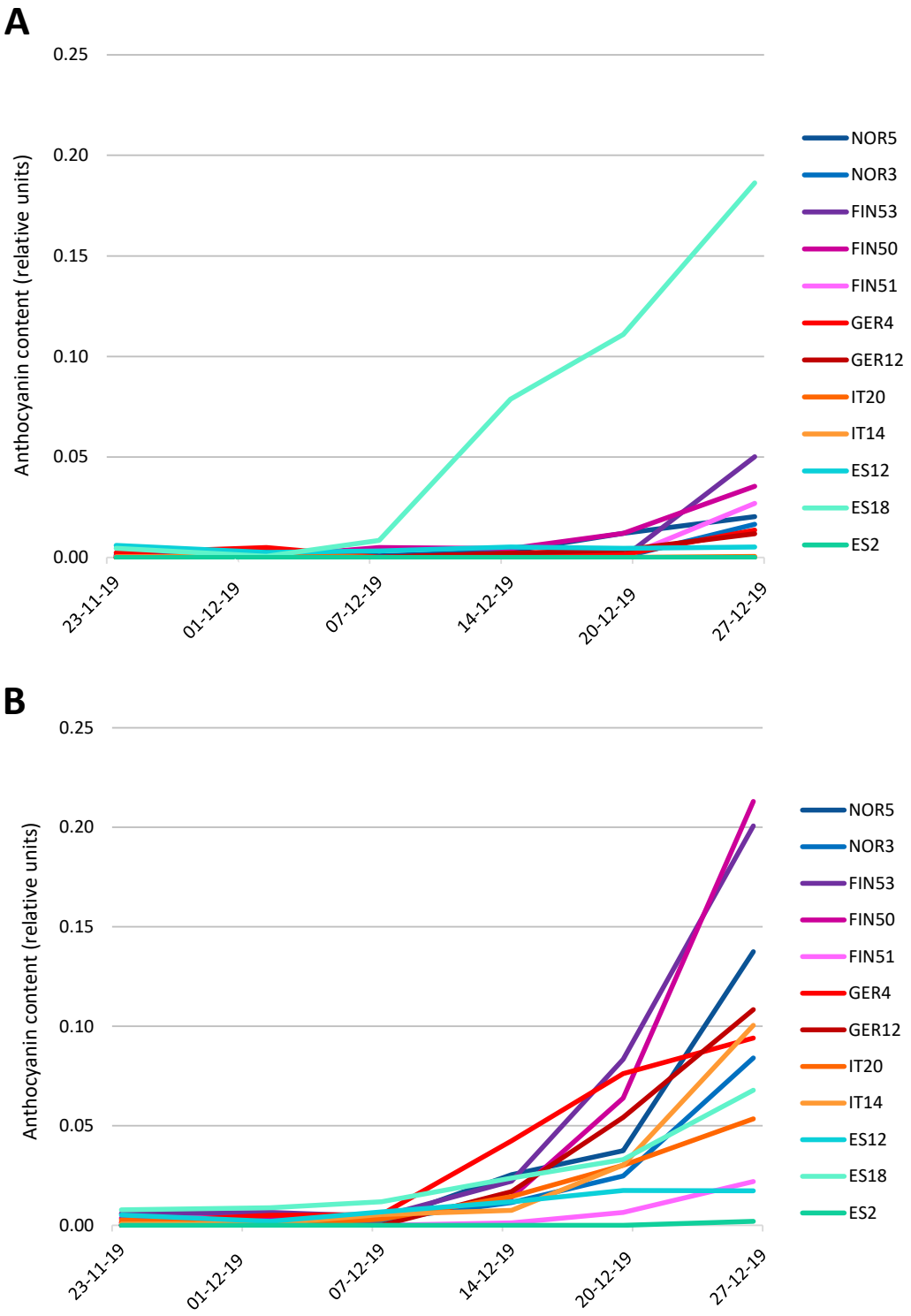


Figure 19: *The winter leaf anthocyanin content of twelve Fragaria vesca genotypes in two different temperature treatments. A: Winter leaf anthocyanin content in the warm room, measured from 23 November 2019 to 27 December 2019. B: Winter leaf anthocyanin content in the cold room, measured from 23 November 2019 to 27 December 2019.*

In the cold room (Figure 19 B) the winter leaves of genotypes FIN53 and FIN50 produced the highest anthocyanin levels, slightly above 0.2 on the last day of the measurements. Genotype ES2 remained very close to zero and the Finnish genotype FIN51 had low values together with ES12. However, all genotypes except ES2 in the cold room increased their anthocyanin values over time.

5. Discussion

5.1 Summer and Winter Leaf Size

Because the genotypes used in this study represented a wide geographical range, the assumption was that their summer and possible winter leaves would show differences in their size. Based on previous research concerning *Fragaria vesca* (Åström et al. 2015, Still 2019) it could also be expected that any winter leaves would be smaller than the summer leaves of the same genotype. According to the measurement results, both of these assumptions turned out to be true, except for genotype ES18 where middle leaflet length, middle leaflet width and petiole length in summer and winter leaves were relatively similar. However, this difference can be explained by the fact that during observations of the same plant individuals for the purposes of another study after December 2019, it became evident that in genotypes ES18 and ES12 what were assumed to be winter leaves in November 2019, were not winter leaves after all. Thus, even the colder temperature treatment did not induce winter leaf development in these two genotypes during the experiment period. In a previous study by Still (2019), genotype ES12 did produce winter leaves, but the study in question was executed in field conditions with even colder temperatures.

Since the Finnish genotypes FIN50, FIN51 and FIN53 have been collected from South Finland and their growth places are located close to each other, similarities could have been expected. Nevertheless, genotype FIN50 stood out in summer leaf size measurements, but these differences were no longer evident in winter leaves. Thus, FIN50 had larger summer leaves than the other Finnish genotypes, but this difference did not transfer to winter leaves. In a previous study by Still (2019) with ten *Fragaria vesca* genotypes, FIN50 kept its summer leaves longer than the other Finnish genotype in the study, so in addition to leaf size there was also a difference in developmental response. Larger summer leaves could be connected to higher photosynthetic capacity. In general, leaf size differences in *Fragaria vesca* genotypes manifest their adaptation to local conditions.

For plants, drought is one of the risks of winter (Marchand 2014, Salonen 2006) and closely connected to cold-induced ice-crystal formation (Chalker-Scott 1999, Marchand 2014). According to Åström et al. (2015), small winter leaf size has a connection to drought resistance because in smaller leaves there is less room for transpiration. The Norwegian and Finnish *Fragaria* genotypes come from areas where winters tend to be harsher than generally in Germany, Italy and Spain, and their winter leaf size appears to comply with the drought resistance idea because in all measurements (middle leaflet width, middle leaflet length and petiole length), the Norwegian and Finnish winter leaves were smaller than in the other genotypes. The only anomaly to this was winter leaf petiole length in the warm treatment, where the petioles in Finnish genotypes were longer than the petioles of Italian genotypes and GER4.

Shorter petioles in winter leaves may allow *Fragaria vesca plants* to benefit from warmer conditions closer to the ground, thus reducing the risk of frost damage. Furthermore, in areas with snow in the winter, e.g. Norway and Finland, small winter leaf size places the rosette in the zone between ground and snow cover, where carbon dioxide levels are also higher and promote photosynthesis. (Still 2019) The Norwegian and Finnish winter leaf petioles in this study support this idea too, and so do the petioles of genotype ES2 with its short winter leaf petiole in the cold treatment. ES2 had the highest altitude of all genotype origins, so it may experience cold winter temperatures.

The second hypothesis regarding leaf size was that temperature and/or genotype do influence winter leaf size. Based on statistical tests, this hypothesis was also correct. Figures 5, 6 and 7 show that the winter leaf middle leaflet width, middle leaflet length and petiole length were generally smaller in the cooler room. Therefore, in addition to genotypes being different, temperature affects the size of the developing winter leaf. Nevertheless, as could have been expected due to their altitudinal origins, the genotypes' leaf size measurements did not follow a latitudinal order.

In leaf senescence observations, the early summer leaf senescence in the warm room was an interesting result because colder conditions could have been expected to induce summer leaf senescence sooner. However, in warmer conditions plants usually have a better energy balance and ability to grow. Therefore, with good energy resources their leaf production is faster, and the leaf life cycle can be shorter. The photosynthetic capacity of *Fragaria vesca* summer leaves is not optimal in winter conditions, which is why the plants produce better adapted winter leaves. In the cooler room the energy balance of the plants may have not been as good, and thus they could not allocate resources to winter leaf production as quickly as the *Fragaria vesca* in the warm room.

5.2 Stolon Development

Stolon development in *Fragaria vesca* is connected to the production of clones, which are a mode of asexual reproduction (Zhang & Zhang 2007). According to a study conducted by Schulze et al. (2012), the production of clones in *Fragaria vesca* is connected to maintaining local populations rather than establishing new populations. Dispersal for new populations is rather achieved with seeds that are spread by endozoochory (Schulze et al. 2012). Added ecological benefits of clones include better ability for resource foraging by spreading and also decreased mortality risk (Barrett 2015). Naturally, the success of clones is dependent on the surrounding environmental conditions, just as the success of seeds is. The production of stolons and clones is also energetically expensive. The combination of low likelihood of survival or establishment and the loss of energy would explain why *Fragaria vesca* would stop stolon production as winter approaches. On the other hand, for the southern genotypes that experience only mild winters or no winters at all, the conditions

could still promote stolon production. Of course, this is not only dependent on latitude, but also on altitude, since winter conditions in mountainous areas can be similar to those in high latitudes.

The results from this study show that genotype ES2 was the only one in both warmer and cooler rooms to stop stolon production at the same time, and it was also among the earliest in both rooms. This genotype had the highest altitude of all the origins and perhaps experiences harsher conditions in the winter than the other southern genotypes. The response of this genotype shows indifference to temperature conditions, indicating that some other mechanism than temperature controls its stolon production. In addition to cold temperatures, light availability has also been shown to affect acclimation (Crawford 2014, Marchand 2014)

The opposite of ES2 in stolon development behavior was genotype ES12, which kept producing stolons throughout the experiment period in both rooms. Thus, it was also indifferent to temperature conditions. Moreover, as already mentioned, it was one of the two genotypes that did not produce winter leaves during the experiment period, which might partly explain why it continued producing stolons.

Genotype FIN51 had its last stolons a week earlier in the warm room than in the cooler room, but a week of discrepancy is not necessarily significant. Because the *Fragaria vesca* plants in both temperature treatments did not stop their stolon production at the same time, the hypothesis stating that genotype does have an effect on when *Fragaria vesca* stop producing stolons appears to be correct. Furthermore, according to the results, cooler temperature did affect both the number of stolons that the *Fragaria vesca* genotypes produced and the time when stolon production ended. Genotypes NOR5, NOR3, FIN53, GER4 and ES18 continued their stolon production longer in the warmer room. The results are similar as those described by Chabot (1978), who reported that vegetative reproduction in *Fragaria vesca* favors warmer conditions. Nevertheless, genotype ES2 shows that these results were not uniform for all genotypes.

5.3 Summer and Winter Leaf Chlorophyll Content and Treatment Differences

The chlorophyll level of senescing leaves decreases in plants that attempt to recover this valuable pigment. In a study by Åström et al. (2015), a decrease of chlorophyll content was found to be the first sign of summer leaf senescence in *Fragaria vesca.* In this study, the chlorophyll level of summer leaves in both temperature treatments decreased as a function of time, but more rapidly in the warm room. Thus, the hypothesis that summer leaf chlorophyll content varies according to temperature holds true. Moreover, there were also differences between genotypes at the same temperature and within a single genotype at different temperature treatments. However, the summer leaves in the warmer room started senescing sooner than in the cooler room. The early onset of leaf senescence and rapid loss of chlorophyll content in the warm room summer leaves of FIN53, FIN51, ES2 and ES12 was a clear difference compared to the other genotypes. In genotypes ES2 and FIN51 this was also accompanied with the end of stolon production.

As could be expected based on previous studies by Åström et al. (2015) and Still (2019), in this study the chlorophyll content of the winter leaves remained relatively high and temperature did not seem to have an effect on the chlorophyll levels. However, a surprising phenomenon was that the genotype ES18 winter leaf chlorophyll content in the warmer room began decreasing. An explanation for this might be the later discovery of the leaves in question not being winter leaves after all, but this does not fully explain why the same did not happen in the cooler room. It might be connected to better energy balance in the warm room, which promotes more frequent leaf renewal. Nevertheless, ES12 that did not produce winter leaves either, did not show a chlorophyll decrease in the warm room like ES18. Overall, the winter leaf chlorophyll content results support the hypothesis assuming differences between genotypes. Within genotypes the hypothesis is not entirely accurate because many genotypes, e.g. ES2, ES18, ES12, FIN51 had similar winter leaf chlorophyll content in both temperature treatments.

5.4 Summer and Winter Leaf Flavonol and Anthocyanin Content and Treatment Differences

Secondary compound production, e.g. increase in flavonol and anthocyanin content, is connected to stress responses in plants (Wink 2010). Such a stress occurs, for example, when senescing leaves become vulnerable to photo-oxidative damage (Hoch et al. 2001). In this study *Fragaria vesca* genotypes produced both flavonols and anthocyanins, but there were differences in the summer and winter leaf values of these compounds. Moreover, to some extent the flavonol and anthocyanin content in summer leaves did differ between genotypes at the same temperature and within genotypes at different temperature treatments. The same holds true for winter leaf flavonol and anthocyanin content, and thus both hypotheses turned out to be correct.

In summer leaves the flavonol content fluctuated in both temperature treatments, but the values in the cooler room generally increased, except in genotype ES2, where the summer leaves senesced early. In the warmer room summer leaf flavonol content a general tendency towards increase or decrease could not be detected. In winter leaves the flavonol content showed a steady, slow increase as a function of time in all genotypes in both temperature treatments. In cooler temperature treatments the flavonol content of the leaves increased both in summer and winter leaves. The increase in cold room summer leaf flavonol content is most likely a response to cold stress. Oddly, however, this response is not seen in the winter leaves of ES12 and ES18 that turned out to be summer leaves. A possible explanation for this might be that these leaves had also produced anthocyanins that could have made flavonols redundant as a response to cold.

The summer leaf anthocyanin content fluctuations reflect the transient nature of these compounds. The anthocyanin levels of summer leaves in the warm room were mostly low and lower than in the cool room, where they increased unless the leaves were dead. In winter leaves the anthocyanin levels expressed a weak increase in the warm room, and in the cold room the anthocyanin levels increased in most genotypes.

However, the anthocyanin levels of the winter leaves remained mostly at a lower level than in the summer leaves. These differences can be explained by the fact that anthocyanins have been connected to both chlorophyll recovery in senescing leaves (Hoch et al. 2001, 2003, Feild et al. 2001) and cold acclimation (Christie et al. 1994). Therefore, summer leaf senescence in this study most likely induced anthocyanin production to protect the photosynthetic apparatus and cooler temperature heightened their production even more. In winter leaves the anthocyanin production is probably linked to lower temperatures. The higher and increasing anthocyanin levels of the Spanish genotype ES18 winter leaves in the warmer room can be explained by the fact that the measured value is from a summer leaf.

5.5 Sources of Error

When considering the validness of the results obtained from this study, it is important to note that the current experimental setup had to comply with the facilities available. Thus, the space in the greenhouses and the cooling capacity of the greenhouse rooms set boundaries for what could be done. A possible source of error is temperature in the greenhouse rooms. During the last four weeks there were some inconsistencies in the temperature of the cooler room due to changing temperatures and weather conditions outside the greenhouse and the mechanics of the room. For example, during one particularly stormy day some of the roof shutters did not close properly and some heavy rain fell upon the plants. On 11 December 2019 the temperature log of the greenhouse shows an increase in the colder room. On this day, during the afternoon the temperature rose up to +17°C for a few hours. This might explain the small peak in the winter leaf chlorophyll content on the 14 December 2019 measurement results.

In addition to temperature adjustments, the plant arrangement in the greenhouse could have been different from what it was. Now the arrangement of the plants was random in the sense that the different genotypes were mixed, but each plant individual stayed in the same spot for most of the time during the measurement period. Thus, plant individuals located on the side of the table that was facing the greenhouse walls may have been more exposed to sunlight that was coming through the wall panels, or plant individuals located closest to the door of the room may have been exposed to slight temperature fluctuations. However, constant changing of the placement of the plants would have complicated performing the measurements and made them even more time-consuming. Moreover, the overall results for each genotype are still fairly reliable because there were several plant individuals of each genotype and they were located in different positions on the tables.

Finally, the initial planting of the *Fragaria vesca* could have taken place earlier in the growing season. This way all the plants would have been fully grown before the experiment was started. Now the clones obtained from genotype ES18 were smaller than the clones of other genotypes, which could have affected the results,

especially in summer leaf size measurements. Furthermore, the accuracy of the winter leaf Dualex measurement results for the Norwegian and Finnish genotypes may have suffered from the observed chlorosis/necrosis appearing on the leaves towards the end of the experiment.

6. Conclusions

Based on the results of this study, it is evident that *Fragaria vesca* genotypes express differences in ecophysiological processes as a result of their origin, and these differences are adaptations to local conditions. The results show that temperature does have an effect on *Fragaria vesca* winter leaf development. Moreover, cooler temperature does affect stolon production in some genotypes, but not in a similar fashion in all of them. Temperature also affects the production of secondary compounds. Colder conditions are a stress and *Fragaria vesca* plants react by producing more flavonols and anthocyanins as a response. Moreover, leaf senescence, which can be seen as lowering levels of chlorophyll, also increases anthocyanin content. However, the results also indicate that some other mechanism besides temperature controls the ecophysiological processes of *Fragaria vesca* acclimation.

In order to understand fully the mechanisms of seasonal leaf dimorphism in *Fragaria vesca*, more research should be conducted. A possible next step would be to test the effect of photoperiod on winter leaf development in different *Fragaria* genotypes. Future research could also include more observations of the genotypes, especially the southern ones, in field conditions at higher latitudes. Moreover, since climate change is affecting the likelihood of snowfall in winter, particularly in southern Finland where the Finnish genotypes of this study have been collected, it would be interesting to test whether a cover of snow or the lack of it have an effect on the overwintering strategy and success of *Fragaria vesca*.

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